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(54) Title: IMMUNOCHEMICAL LOCALIZATION OF HEPARANASE IN MOUSE AND HUMAN MELANOMAS

(57) Abstract

Antibodies directed against N-terminal heperanase peptide are produced. These antibodies are used for detection of heparan sulfate endoglycosidase in human and murine tumors. Localization and mounts of these heparanase antigens may ultimately be used to design appropriate therapeutic courses.

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IMMUNOCHEMICAL LOCALIZATION OF HEPARANASE
IN MOUSE AND HUMAN MELANOMAS

5   The United States government retains rights in the
present invention because of sponsorship by National
Institute of Health Grants such as RO1-CA 41524, R35-
CA44352 and P30-CA 16672.

10   CROSS REFERENCES TO RELATED APPLICATION

        This application is a continuation-in-part
application of copending U.S. patent application serial
number 377,015 filed July 7, 1989, which is a divisional
15   of U.S. patent application serial number 012,860 filed
February 20, 1987, which is a continuation-in-part of
serial number 839,890, filed March 10, 1986.

        The immunochemical localization of the glycosamino
20   endoglycosidase, heparanase, in human and murine tumor
types is an object of the present invention.

        The present invention also relates to an assay for
endoglycosidase enzymic activity and a labeled substrate
25   for use in such an assay. The assay of the present
invention is viewed as useful for the detection of
cancerous malignancies.

        A class of biological substances called the pro-
30   teoglycans form the ground substance in the extracellular
matrix of connective tissues. These proteoglycans are
polyanionic substances of high molecular weight and
contain many different types of heteropolysaccharide side
chains covalently linked to a polypeptide backbone.
35   These proteoglycans may contain over 95% carbohydrates.
The polysaccharide groups of the proteoglycans were
formerly called mucopolysaccharides but now are
preferably termed glycosaminoglycans since all contain
derivatives of glucosamine or galactosamine.

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A variety of enzymes may be involved in the normal metabolic degradation of proteoglycans. Initial proteo-glycan degradation often involves proteolysis to separate

5   or digest protein components. Such proteolysis results in the production of glycosaminoglycans. The glycosaminoglycans in turn are subject to glycosamino-glycan endoglycosidase enzymic action which produces smaller glycosaminoglycan fragments. The glycosamino-

10 glycans or fragments thereof are subject to glycosamino-glycan exoglycosidase enzymic action which produces monosaccharides from the non-reducing ends of glycosaminoglycans.

15 An increasing interest in the endoglycosidases has arisen in recent years because of a possible relationship of these enzymes with tumor invasiveness and tumor meta-static activity. Nicolson (1982, Biochem. Biophys. Acta. V 695, pp 113-176) reviewed a variety of oligosaccharide-

20 degrading enzymes (pp 141-142) reported to be of interest in malignant disease. Nicolson (1982, J. Histochem. & Cytochem. V 30, pp 214-220) described a proposed mechanism for tumor cell invasion of endothelial cell basal lamina and a related production of degradation

25 products from proteins and glycosaminoglycans. Kramer et al. (1982, J. Biol. Chem. V 257, pp 2678-2686) reported a tumor-derived glycosidase capable of cleaving specifically glycosaminoglycans and releasing heparan sulfate-rich fragments.

30

Irimura et al. (1983, Analyt. Biochem. V 30, pp 461-468) describe high-speed gel-permeation chromatography of glycosaminoglycans. Heparan sulfate degrading activity of melanoma cells was measured by using this

35 chromatographic procedure. Nakajima et al. (1983, Science, V 220, pp 611-613) described a relationship of

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metastatic activity and heparan sulfate degrading activity in melanoma cell lines. The disappearance of higher molecular weight heparan sulfate was followed by polyacrylamide gel electrophoresis, staining and
5   densitometry.

Vlodavsky et al. (1983, Cancer Res. V 43, pp 2704-2711) described the degradation by two T-lymphoma cell lines of ^{35}S labeled proteoglycans from confluent endo-
10   thelial cells. The highly metastatic line had much higher ^{35}S liberating activity than did the low metastatic line.

Irimura et al. (1983, Proc. Am. Soc. Cancer Res. V
15   24, p 37, abstract 144), using high performance liquid chromatography, describe heparan sulfate degradative enzyme activity of melanoma cells. Nakajima et al (1984, J. Biol. Chem. V 259, pp 2283-2290) describe characterizations of metastatic melanoma heparanase.
20   High speed gel permeation chromatography and chemical analyses were used in a description of functional substrates and products formed.

The background described herein involves an interest
25   in convenient, accurate and reproducible endoglycosidase assays, particularly since endoglycosidases may play critical roles in the establishment of tumor metastases.

The ability of tumor cells to invade host tissues
30   and metastasize to distant, often specific organ sites, is one of their most important properties. Metastasis formation occurs via a complex series of unique interactions between tumor cells and normal host tissues and cells. These processes involve several discrete and
35   selective steps such as: invasion of surrounding tissues, penetration of lymphatics of blood vessels and

transport in lymph or blood, or dissemination into a
serous cavity, arrest and invasion at distant sites, and
survival and growth to form secondary lesions.

5   Basement membranes are continuous sheets of extra-
cellular matrix composed of collagenous and non-
collagenous proteins and proteoglycans that separate
parenchymal cells from underlying interstitial connective
tissue. They have characteristic permeabilities and play
10   a role in maintaining tissue architecture. Metastasizing
tumor cells must penetrate epithelial and endothelial
basement membranes during invasion and metastasis, and
the penetration and destruction of basement membranes by
invasive tumor cells has been observed using electron
15   microscopy. Since basement membranes are rigid
structures formed from unique sets of macromolecules,
including type IV collagen, laminin, heparan sulfate
(HS), proteoglycan and fibronectin, the successful
penetration of a basement membrane barrier probably
20   requires the active participation of more than one tumor
cell-associated enzyme.

        Due to its unique physical and chemical properties
such as its polyanionic character and barrier properties
25   against macromolecules (Kanwar et al., 1980 J. Cell.
Biol. V 86, pp 688-693), heparan sulfate (HS) is an
important structural component of basement membranes. HS
binds to fibronectin, laminin and type IV collagen, and
these molecules have been collectively observed in the
30   basal lamina using antibodies raised against each
component. HS may be involved in basal lamina matrix
assembly by promoting the interactions of collagenous and
non-collagenous protein components while protecting them
against proteolytic attack. Thus, the destruction of HS
35   proteoglycan barrier could be important in basement
membrane invasion by tumor cells.

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The interactions between malignant cells and
vascular endothelium have been studied using monolayers
of cultured vascular endothelial cells that synthesize an
extracellular matrix resembling a basement membrane.
5   With this model, it has been found that metastatic B16
melanoma cells degrade matrix glycoproteins, such as
fibronectin, and matrix sulfated glycosaminoglycans, such
as heparan sulfate. Since heparan sulfate was released
in solution as fragments approximately one-third their
10   original size, it has been proposed that metastatic tumor
cells characteristically have a heparan sulfate
endoglycosidase.

15   The relation between metastatic properties and the
ability of five B16 melanoma sublines of various
implantation and invasion characteristics to
enzymatically degrade subendothelial extracellular matrix
indicated that highly invasive and metastatic B16
20   sublines degraded sulfated glycosaminoglycans faster than
did sublines of lower metastatic potential (Nakajima et
al., (1983), Science V 220, p 611), and intact B16 cells
(or their cell-free homogenates) with a high potential
for lung colonization also degraded purified heparan
25   sulfate at higher rates than did B16 cells with a poor
potential for lung colonization (ibid). The abilities of
B16 cells to degrade HS from various origins and other
purified glycosaminoglycans (heparin, chondroitin 4-
sulfate, chondroitin 6-sulfate, dermatan sulfate, keratan
30   sulfate, and hyaluronic acid) has been studied. In order
to analyze glycosaminoglycan degradation products, an
analytic procedure was developed using high-speed gel
permeation chromatography (Irimura et al., (1983) Anal.
Biochem. V 130, p 161; Nakajima et al., (1984) J. Biol.
35   Chem. V 259, p 2283). HS metabolically labeled with
[^{35}S]sulfate was purified from basement membrane producing

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EHS sarcoma and PYS-2 carcinoma cells, and subendothelial
matrices of bovine aortic endothelial (BAE) and corneal
endothelial (BCE) cells (ibid). HS molecules purified
from bovine lung and other glycosaminoglycans were
5   labeled with tritium at their reducing termini using
NaB[^{3}H]$_4$. These labeled glycosaminoglycans were incubated
with B16 cell extracts in the absence or presence of D-
saccharic acid 1,4-lactone, a potent exo-β-glucuronidase
inhibitor, and degradation fragments were analyzed by
10   high-speed gel permeation chromatography. HS isolated
from the various origins described above were all
degraded into fragments of characteristic molecular
weight, in contrast to hyaluronic acid, chondroitin 6-
sulfate, chondroitin 4-sulfate, dermatan sulfate, keratan
15   sulfate, and heparin, which were essentially undegraded.
Heparin, but not other glycosaminoglycans, inhibited HS
degradation. The time dependence of HS degradation into
particular molecular weight fragments indicated that
melanoma heparanase cleaves HS at specific intrachain
20   sites (ibid). In order to determine specific HS cleavage
points, the newly formed reducing termini of HS fragments
were investigated by: labeling with NaB[^{3}H]$_4$; hydrolysis
to monosaccharides; and analysis of these saccharides by
paper chromatography. Since ^{3}H-reduced terminal mono-
25   saccharides from HS fragments were overwhelmingly (>90%)
L-gulonic acid, the HS-degrading enzyme responsible was
an endoglucuronidase (heparanase).

HS-degrading endoglucuronidases have been found in
30   various tissues, such as human skin fibroblasts, rat
liver cells, human placenta, and human platelets. HS-
degrading endoglucuronidases in mammalian cells were
reported previously by other investigators to be
"heparitinases" to indicate heparitin sulfate (heparan
35   sulfate)-specific endoglycosidase. However, heparitinase
originally was used to designate an elimination enzyme

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(EC 4.2.2.8) in _Flavobacterium_ _heparinum_, and this enzyme
cleaves nonsulfate and monosulfated 2-acetoamido-2-deoxy-
alpha-D-glucosyl-D-hexuronic acid linkages of HS.   Since
HS-specific endoglycosidases in mammalian cells are endo-
5   glucuronidases, except for one found in skin fibroblasts,
it was proposed that mammalian cell endoglucuronidases
capable of degrading HS should be called "heparanases",
consistent with the currently used term "heparan
sulfate".
10

Glycosaminoglycan endoglycosidases have been assayed
for enzyme activity by some other means.   For example,
Oldberg et al. (1980, Biochem. V 19, pp 5755-5762)
described an assay for a platelet endoglycosidase which
15   degraded heparin-like polysaccharide.   This assay
involved measuring a decreasing amount of ^{3}H-heparan
sulfate, the decrease being a function of endoglycosidase
activity.

20   Endoglycosidase assays using solid-phase substrates
were described by Iverius (1971, Biochem. J. V 124, pp
677-683) and Oosta et al. (1982, J. Biol. Chem. V 257, pp
11249-11255).   Iverius coupled a variety of glycosamino-
glycans to cyanogen bromide-activated Sepharose 4B beads.
25   In one case the endoglycosidase hyaluronidase was assayed
for enzymic activity by incubation of the enzyme with
chondroitin sulfate bound to Sepharose 4B.   The enzyme
activity was monitored by following the production of
soluble uronic acid with a colorimetric assay procedure.
30   Oosta et al. described an assay for heparitinase, an
endoglycosidase from platelets which cleaves heparin and
heparan sulfate.

The Oosta et al. system and assay comprised:

35

(1)   Coupling heparin with N-succinimide 3-(4-

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hydroxylphenyl) propionate.

(2)   Labeling the coupled heparin by incubation with
       $Na^{125}I$ and chloramine-T.

5

(3)   Coupling the ^{125}I heparin to cyanogen bromide-
       activated beads of Sepharose 4B, and

(4)   Incubating the endoglycosidase with the ^{125}I-
10   heparin coupled to Sepharose 4B beads and
       measuring solublized radioactivity.

     In these two methods, glycosaminoglycans were cross-
linked to agarose by the reaction of free amino groups of
15 glycosaminoglycans and amino-reactive cyanogen bromide-
activated agarose.   Since glycosaminoglycans, such as
heparin and heparan sulfate, have several free
glucosamine amino groups, this type of crosslinking
results in excessive covalent linkages between substrate
20 molecules and agarose gel, resulting in a loss of
susceptibility to endoglycosidases and nonlinear rates of
degradation.   Thus the most desirable solid-phase
substrate for glycosaminoglycan endoglycosidase is
glycosaminoglycan crosslinked to a solid support at one
25 end of the molecule such as reducing terminal.

     Heparanase, an endo-β-D-glucuronidase, has been
associated with melanoma metastasis.   Polyclonal
antibodies directed against the murine N-terminal
30 heparanase peptide detected a M_r-97,000 protein on SDS-
polyacrylamide gel electrophoresis of mouse melanoma ad
human melanoma cell lysates.   In an indirect
immunocytochemical study, human A375-SM and mouse B16-BL6
melanoma cells were stained with the anti-heparanase
35 antibodies.   Heparanase antigen was localized in the
cytoplasms of permeabilized melanoma cells as well as at

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the cell surface of unpermeabilized cells.
Immunohistochemical staining of frozen sections from
syngeneic mouse lungs containing micrometastases of B16-
BL6 melanoma demonstrated heparanase localized in
5   metastatic melanoma cells. Similar studies using frozen
sections of malignant melanomas sesected from patients
indicated that heparanase is localized in invading
melanoma cells. Studies relating to the present
invention suggest that (a) the N-terminus of the
10 heparanase molecule in mouse and human is antigenically
related; (b) heparanase antigens are localized at the
cell surface and in the cytoplasm of metastatic human and
mouse melanoma cells; and (c) heparanase antigens are
enriched in invasive and metastatic murine and human
15 melanomas in vivo. Although polyclonal antibody
preparations have been used herein, it is understood that
monoclonal antibodies or a mixture of monoclonal
antibodies with similar specificity may be also produced
by well-known means and used analogously as desired.

20

    A solid phase substrate which yields soluble labeled
products upon hydrolysis by a glycosaminoglycan
endoglycosidase and methods of producing said substrate
are comprised in the present invention. The solid phase
25 substrate comprises glycosaminoglycan bearing labeled N-
acetyl groups and being reductively aminated at its
reducing terminal end to produce an amine-terminus. The
substrate is further coupled to an amino-reactive solid
matrix through its amine-terminus.

30

    A method of producing the solid phase substrate
comprises the steps of: at least partially N-desulfating
or N-deacetylating a glycosaminoglycan; labeling at least
partially N-deacetylated or N-desulfated glycosamino-
35 glycan to produce labeled glycosaminoglycan; completely
N-acylating the labeled glycosaminoglycan with acyl

anhydride or acyl halide; reductively aminating a
reducing terminal end of said labeled glycosaminoglycan
to produce labeled amine-terminal glycosaminoglycan; and
coupling, through its terminal amine, the labeled amine-
5   terminal glycosaminoglycan to an amine-reactive solid
phase support to produce the solid matrix substrate.

The labeling may be accomplished by substitution on
amino groups of the partially N-desulfated or N-
10   deacetylated glycosaminoglycan of a substance yielding a
detectable signal. This substance may be a radioisotopic
label, a fluorescent label or an enzymatic label. A
fluorescent label is preferred for ease of assay and a
radioisotopic label for similarity to the natural
15   glycosaminoglycan.

Fig. 1. - Synthesis of a solid-phase heparanase
substrate: Chemical modification and radiolabeling of HS
and its coupling to amino-reactive agarose gel bead.
20

Fig. 2. - Elution profiles on high-speed gel
permeation chromatography of unmodified- and chemically
modified-HS before and after treatment with B16 melanoma
heparanase. HS, heparan sulfate; NDS-HS, N-desulfated
25   heparan sulfate NDS-HAc-HS, N-desulfated N-acetylated
heparan sulfate. These glycans labeled with tritium at
the reducing termini (open circles) and their fragments
produced by the incubation with B16 melanoma-cell
extracts in the presence of saccharic acid 1,4-lactone
30   (SAL) (solid circles), were chromatographed on two
sequential 0.7 x 75-cm columns of Fractogel-TSK HW-55(S)
with 0.2 M sodium chloride at a flow rate of 1.0 ml/min
at 55°C. Arrows (a)-(e) indicate the elution positions
of the standard glycans: (a) C6S from shark cartilage (M_r
35   60,000); (b) HS from bovine lung (M_r 34,000); (c)
heparin from porcine mucosal tissue (M_r 11,000); (d)

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monosialosyl biantennary complex-type glycopeptide from
porcine thyroglobulin (M_r 2190); (e) N-acetyl-D-
glucosamine (M_r 221).

5   Fig. 3. - Dose dependent degradation of partially N-
desulfated N[^{14}C]acetylated heparan sulfate (PNDS-N[^{14}C]Ac-
HS) immobilized on agarose by B16 melanoma cell
heparanase. The PNDS-N[^{14}C]Ac-HS (4500 cpm) immobilized
on agarose was incubated with various amount of B16 cell
10   extract for 6 h (open squares), 12 h (open circles), and
24 h (open hexagons), or with various amounts of heat
inactivated (100°C, 5 min) B16 cell extract for 12 hr
(open triangles) in the presence of SAL. The released
radioactivity in a half volume of the supernatant versus
15   the amount of cell extract added (ug protein) was
plotted.

        Fig. 4 shows the levels of heparanase activity in
the sera of controls and patients with malignant
20   melanoma.

        Fig. 5 shows levels of heparanase activity in sera
of rats injected with a highly metastatic adenocarcinoma.

25   Fig. 6 shows the relationship between serum
heparanase activity and the size of a primary metastatic
tumor in rats.

        Fig. 7 shows the relationship between rat serum
30   heparanase levels and numbers of metastases from a
malignant tumor.

        Fig. 8 shows the position of substrate hydrolysis
for melanoma heparanase.

35
        Figure 9 - Autoradiograph of a 7.5% SDS-

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polyacrylamide gel containing proteins immunoprecipitated
from human A375-SM (a-d) and mouse B16-BL6 (e-h) melanoma
cell lysates metabolically labeled with [^{35}S]-methionine.
Cell lysates were incubated with PBS (d,h), biotinylated

5   anti-heparanase antibodies (b,c,f,g), or biotinylated
anti-heparanase antibodies preincubated with heparanase-
peptide (a,e). They were subsequently incubated with
streptavidin-agarose (a-h) and samples were run in the
presence of ß-mercaptoethanol. The molecular weight

10   markers at the far right are expressed in kDa.

Figure 10 - Micrographs of the immunocytochemical
localization of heparanase in human A375-SM melanoma
cells. Cultured cells were fixed (a-f), or permeablized

15   (a,c-f). They were then incubated with anti-heparanase
antibodies (a,b,e), with anti-heparanase antibodies
preabsorbed with heparanase peptide (d) or with PBS
(c,f). The cells were then washed and incubated with
peroxidase-conjugated goat-anti-rabbit IgG (a,b,d,f) or

20   PBS (c,e). Original magnification x25; bar=30 µm. The
micrographs are of the same magnification.

Figure 11 - Immunohistochemical localization of
heparanase in a human cutaneous melanoma (a,b,c) or

25   murine melanoma metastasis in the lung (d,e,f). Frozen
sections fixed with 2% paraformaldehyde were stained with
hematoxylin and eosin (a,d) or incubated with anti-
heparanase antibodies (c,f) or anti-heparanase antibodies
preabsorbed by heparanase peptide (b) or PBS (e). The

30   sections were then incubated with goat-anti-rabbit IgG
conjugated with peroxidase (b,c,e,f). Original
Magnification x80; bar=20 µm. The micrographs are of the
same magnification.

35   Immunoassays, such as those using antibodies raised
to an glycosaminoglycan endoglycosidase such as heparan

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sulfate endoglycosidase, for example, are described, which measure the enzyme. The present invention also describes a new assay for glycosaminoglycan endoglycosidase activity, most preferably that of the

5   heparan sulfate endoglycosidase termed "heparanase". This new assay describes using a solid phase substrate which yields soluble labeled products upon hydrolysis by a glycosaminoglycan endoglycosidase. The new assay also describes novel adaptations of this solid phase enzymatic

10   assay to liquid-phase conditions.

Among its many functions, the extracellular matrix is a tissue barrier that tumor cells must penetrate in the process of tumor invasion and metastasis.

15   Extracellular matrix includes basement membranes and connective tissue stroma and is composed of collagens, proteoglycans, laminin, fibronectin and other glycoproteins. Degradation of this matrix, an important step in tumor cell invasion and metastasis, is mediated

20   by a variety of degradative enzymes, including proteases and glycosidases of tumor or host cell origin (Mullins et al., *Biochim. Biophys. Acta*, **695**, 177-214 (1983); Nakajima et al., *J. Cell. Biochem.*, **36**, 157-167 (1988); Nicolson, *Biochim. Biophys. Acta*, **695**, 113-176 (1982);

25   Liotta, *Cancer Res.*, **46**, 1-7 (1986); Sloane et al., *Cancer Met. Rev.*, **3**, 249-263 (1984). One extracellular matrix-degrading enzyme, heparanase, was identified originally in murine and human melanoma cells (Kramer et al., *J. Biol. Chem.*, **257**, 2678-2686 (1982); Nakajima et

30   al., *Science*, **220**, 611-613 (1983). The enzyme was purified and characterized as a glycoprotein of $M_r \sim 97,000$ (Nakajima et al. *J. Cell. Biochem.*, **36**, 157-167 (1988). Levels of heparanase activity have been directly correlated with the lung-colonizing potentials of murine

35   metastatic melanoma cells (Nakajima et al. *Science*, **220**, 611-613 (1983) and human melanoma cells (Nakajima et al.

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Cancer Lett., 31, 277-283 (1986a), and such activity was
found in sera of melanoma patients with metastic disease
(Nakajima et al. *J. Cell. Biochem.*, 36, 157-167 (1988).
Structural analogues of heparanase substrate, such as
5   heparin and its chemically modified derivatives,
inhibited B16 murine melanoma pulmonary metastases in
experimental metastasis assays (Irimura et al.,
Biochemistry, 25, 5322-5328 (1986); Villanueva et al.,
1988).   Therefore, tumor cell heparanase is thought to
10 play an important role in melanoma invasion and
metastasis.

        Although the role of degradative enzymes in the
process of tumor invasion and metastasis has been
15 investigated (Mullins et al. *Biochim. Biophys. Acta*, 695,
177-214 (1983); Liotta et al., *Cancer Res.*, 46, 1-7
(1986); Nicolson, *Biochim. Biophys. Acta*, 695, 113-176
(1982), the localization and sources of these enzymes
have received little attention.   1982), the localization
20 and sources of these enzymes have received little
attention.   Current information on this is largely based
on studies of proteases (Moscatelli et al., *Biochim.
Biophys. Acta*, 948, 67-85 (1988), such as cathepsin B
(Sloane et al., *Cancer Met. Rev.*, 3, 249-263 (1984) and
25 type IV collagenase (Liotta et al., *Nature (Lond.)* 284,
67-68 (1980).   Using biochemical analyses of enzymatic
activities in subcellular fractions, these enzymes have
been associated with tumor cell surface membranes and
various cellular compartments.   Cathepsin B activities
30 were detected in lysosomal and plasma membrane fractions
of B16 murine melanoma cells (Keren et al., Cancer Res.,
48, 1416-1421 (1988); Rozhin *et al.*, Cancer Res., 47,
6620-6628 (1987), and the enzyme was also localized at
the cell surfaces of virus-transformed fibroblasts and
35 other cells (Sylven et al., *Virchows Arch. B Cell
Pathol.*, 17, 97-112 (1974).   Heparanase activities have

been found on melanoma cell surfaces, in cell homogenates
and shedded vesicles of melanoma cells (Nakajima et al.
Science, **220**, 611-613 (1983). Immunochemical studies on
degradative enzymes have provided additional information
5   on their localization and are particularly useful in
identifying the source of an enzyme in tumors that also
contain normal host cells and structures such as
fibroblasts and lymphocytes. Cathepsin B was localized
on some normal cellular components at the invasion front
10 of rabbit carcinoma (Graf et al., *Lab. Invest.*, **45**, 587-
596 (1981).

In accordance with the present invention, anti-
heparanase antibodies directed against the N-terminal
15 heparanase peptides were prepared and characterized.
Using an indirect immunochemical method, heparanase was
localized at the cell surfaces and in the cytoplasm of
human and mouse melanoma cells. The antibodies strongly
stained metastatic melanoma nodules in a murine experi-
20 mental metastasis model and human melanomas from patients
with metastatic disease.

This solid phase substrate comprises a glycosamino-
glycan bearing radioisotopically labeled N-acyl groups.
25 These labeled N-acyl groups are preferably ^3H labeled or
^{14}C labeled acetyl groups although other labeled acyl
groups, such as formyl or propionyl groups may be used.
The solid phase substrate of the present invention may
comprise as the glycosaminoglycan: hyaluronic acid,
30 chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan
sulfate, keratan sulfate, heparan sulfate, heparin, or
combinations thereof. The use of particular glycosamino-
glycans will allow assays for the enzymic activity of
endoglycosidases having a substrate specificity for the
35 particular glycosaminoglycan being used.

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The amino-reactive solid matrix to which the amine-
terminal labeled glycosaminoglycan may be bound may have
many acceptable forms, both in the basic nature of the
matrix and in the amine-reactive chemical site.

5

A preferable solid matrix is agarose-based, most
preferably Sepharose or Sepharose derivatives in bead
form (Pharmacia). Other solid matrices such as
celluloses or polyacrylamides may be used provided that

10   the have amine-reactive substituent functions for coupl-
ing.

It is well known that Sepharose beads may be acti-
vated with cyanogen bromide and then coupled to amine-

15   bearing molecules such as heparin, other glycosamino-
glycans or glycosaminoglycan derivatives. Cyanogen
bromide mediated coupling, is a usable coupling method
for practice of the present invention. Cyanogen bromide
activated agarose or any other amine-reactive solid

20   matrix may couple to more than one amine group of a
glycosaminoglycan and glycosaminoglycan derivative with
multiple amine functions. This multiple coupling to
labeled glycosaminoglycans could lead to insensitive
and/or inaccurate glycosaminoglycan endoglycosidase

25   assays, since a single glycosidase-mediated hydrolytic
event may not result in a soluble product, i.e., a
product not linked to the solid matrix. Thus for the
practice of the present invention, it is of importance
that the labeled glycosaminoglycan derivative to be bound

30   to a solid matrix has but a single primary amino-group.

While a variety of amine-reactive substituents are
known to those skilled in the art, an $\underline{N}$-hydroxy succinide
ester is a preferable amine-reactive function bound to a

35   solid matrix and is commercially available or readily
synthesized. Such $\underline{N}$-hydroxysuccinide esters couple to

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primary amine groups at a pH between about 6 and 9. Aga-
rose may be activated by periodate oxidation to contain
aldehyde functions. This aldehydic agarose may be
reacted with labeled amine-terminal glycosaminoglycan and
5   the linkage stabilized by reduction with sodium
cyanoborohydride. (Perikh et al., Methods in Enzym. Vol
XXXIV, p 81 Acad. Press (1974)). Other commonly used
procedures which may be used to link amine-bearing
labeled glycosaminoglycans to solid matrices include:
10   using a carbodiimide and a carboxyl-bearing solid matrix;
directly reacting the amine-bearing labeled
glycosaminoglycan with a solid matrix bearing a
bromoacetyl, diazonium or epoxy function.

15   The glycosaminoglycans generally have their amine
functions either sulfated or acetylated. After at least
partial N-desulfation or N-deacetylation, for example,
the resultant primary amino groups on the
glycosaminoglycan are available for labelling.
20   Deacetylation may be accomplished by hydrazinolysis under
conditions avoiding excessive alkalinity which could lead
to hybrolysis of glucosaminyl linkages. Desulfation may
be accomplished by formation of pyridinium salts of the
glycosaminoglycan followed by solvolysis in
25   dimethylsulfoxide. Amino group labeling is accomplished
by reaction with a fluorescent compound such as
fluorescein isothiocyanate, an enzyme such as alkaline
phosphatase (and a bifunctional coupling agent) or with a
radioisotopically labeled acyl anhydride or acyl halide.
30   A label is then covalently attached to at least some of
the free amine groups. Remaining free amine groups of
the labeled glycosaminoglycan are then acylated, for
example by acetic anhydride treatment. The acylated
labeled glycosaminoglycan is then aminated at its
35   reducing terminal. This amination is accomplished by
incubation with an amine salt to form a Schiff base with

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the terminal and subsequent reduction to form a terminal
amine.

Amino group labeling may be accomplished by coupling
5   a measurable compound or active protein to at least a few
of the amino groups. The measurable compound may be one
of the many known to be highly absorbent of visible light
or more preferably one which is fluorescent when excited
by irradiation at particular wavelengths such as
10   fluorescein as mentioned above. Labeling by attachment
of enzymes (as alkaline phosphatase mentioned above) as
active proteins to the partially N-desulfated or N-
deacetylated glycosaminoglycan is also a possibility.
Labeling by enzymes or measurable compounds having light
15   absorbent or fluorescent structures, however, may involve
sterically bulky substituents. Such sterically bulky
substituents may, when substituted at too high a level,
render glycosaminoglycan derivatives which are poor
substrates for glycosaminoglycan endoglycosidases. In
20   preliminary experiments, a partially N-desulfated heparan
sulfate was coupled in a 1:1 ratio to fluoroscein
isothiocyanate. This fluorescein labeled derivative was
found to be a good substrate for melanoma heparanase. It
is contemplated that up to a 10:1 ratio of fluorescein to
25   HS may be produced and serve as a heparanase substrate.

One or a very few of these bulky substituents may
not hinder substrate activity and result in good labeled
substrates. Another potential problem with enzyme labels
30   is that enzymes generally contain free amine groups which
may bind to amine-reactive solid matrices. One preferred
label for glycosaminoglycans is a radioisotopic label
similar or identical in structure to naturally occurring
N-substituents. While ^{35}S-sulfate N-substituents could be
35   utilized, ^{14}C- or ^{3}H-acetyl N-substituents are preferred
as readily produced. Although the subsequently described

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substrates and procedures relate primarily to radioiso-
topic labeling there are largely applicable in principle
to other labels, particularly fluorescent labels.

5   When N-radioisotopically labeled glycosaminoglycans
are attached by a single bound at one end to a solid
matrix, a solid phase substrate for a glycosaminoglycan
endoglycosidase is created. As described elsewhere
herein, this solid phase substrate yields soluble radio-
10   isotopically labeled substances as a function of
glycosaminoglycan endoglycosidase enzymatic activity. An
alternative manner of measuring this same activity would
be to observe the disappearance of radioisotopic label
bound to the solid matrix as a function of enzymatic
15   activity. This type of measurement has the disadvantage
of being a negative measurement and also that incubation
supernatant would have to be carefully removed from
residual solid matrix substrate.

20   In a broad sense, the solid phase substrate of the
present invention is one which yields soluble products
labeled with a detectable signal upon hydrolysis by a
glycosaminoglycan endoglycosidase. This solid phase
substrate comprises a glycosaminoglycan bearing a label
25   which does not prevent hydrolysis of the labeled
glycosaminoglycan by a glycosaminoglycan endoglycosidase.
The labeled glycosaminoglycan is linked through a single
end, preferably the reducing terminal end and by a single
covalent linkage, to a solid matrix. The detectable
30   signal may be radioisotopic, light absorbent, fluorescent
or enzymatically active. The solid matrix is preferably
hydrophilic and may include polymers such as cellulose,
dextran, polyacrylates or their derivatives, alone or in
combination. The substrate of the present invention may
35   be soluble if a detectable label is present along with a
tagging molecule. The tagging molecule may be used as a

'handle' for removal of a portion of attached
glycosaminoglycan.

5   The labeling of at least partially desulfated or
deacylated glycosaminoglycan is most preferably accom-
plished by treatment with ^{3}H-acetic anhydride or ^{14}C-
acetic anhydride, although analogous acetyl halides,
particularly chlorides or also alkyl bromides are
contemplated as useful. In addition to other acyl func-
10   tions such as formyl or propionyl, other coupling methods
may be used in this labelling procedure.

       The substrate of this invention may also be a liquid
phase substrate with separation of the cleaved products
15   from the uncleaved substrate occurring after the
enzymatic reaction. In this scheme, a glycosaminoglycan
such as heparan sulfate, for example, could be tagged at
one end, preferably the reducing end, to another
molecule. The glycosaminoglycan should be labeled at
20   additional sites by other molecules such as ^{125}I,
fluorescein, enzymes, and the like, that may be used for
detection of cleaved products in the assay. Among the
advantages available with a liquid substrate of the type
described herein should be an assay with increased
25   sensitivity to the action of glycosaminoglycan
endoglycosidases. This increased sensitivity would at
least in part relate to an enhanced availability in
solution to soluble enzymes.

30   The molecular tag at one end of the
glycosaminoglycan could be either a small molecule, such
as fluorescein or biotin, or a larger molecule, such as a
peptide or a protein. The linkage of this molecule to an
end of the glycosaminoglycan substrate must not
35   significantly inhibit the hydrolysis of the tagged
glycosaminoglycan by the glycosaminoglycan

endoglycosidase. The molecular tag should have the
ability to act as a potential 'handle' for the labelled
glycosaminoglycan chain and for the residue of the
glycosaminoglycan chain remaining after cleavage by a
5   glycosaminoglycan endoglycosidase. As a 'handle', the
molecule would be able to act as a point of attachment
for a protein molecule having affinity for the bound
tagging molecule. Such a protein-molecule relationship
will enable tagged portions of the labeled
10   glycosaminoglycan to be readily separated from labeled
but untagged portions liberated by endoglycosidase-
induced hydrolysis of glycosaminoglycan substrate
hydrolysis. The molecular tag should be either: a) a
haptenic molecule capable of generating specifically
15   binding antibodies when attached to a carrier such as a
protein and immunogenically administered to an animal; b)
a segment of or a whole immunogenic substance such as a
protein or peptide; or c) a substance having a high
binding affinity for existent proteinaceous molecules
20   such as avidin or protein A, for example.

Following incubation with samples containing
endoglycosidase activity, the uncleaved products may then
be separated from the cleaved products by incubation
25   with, for example, solid-phase antibodies having an
affinity for the tag. Proteins other than antibodies
that bind the molecular tag that has been attached to the
end of the glycosaminoglycan may also be used to separate
uncleaved glycosaminoglycan. If solid phase antibodies
30   or solid phase binding proteins are used, the solid phase
may be any support that can be readily coupled or
absorbed to antibodies or binding proteins and that can
affect a separation of cleaved product from uncleaved
substrate. Commonly-used examples of solid phase include
35   agarose; Sepharose; polymers, such as polystyrene; glass;
cellulose and glass beads; and magnetizable beads. The

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solid-phase could be in the form of large or small
particles or a tube or microtiter plate or other device
that is readily adaptable to the detection system.

5   The separation of cleaved from uncleaved
glycosaminoglycan products can also be achieved by an
immunoprecipitation reaction that does not require
antibodies to be linked to a solid phase (see Morgan et
al. (1962) Proc. Soc. of Exp. Biol. Med., V 110, pp 29-
10   35). The precipitating antibodies could be directed
toward the molecule tagged at the end of the
glycosaminoglycan chain.

        To facilitate binding at one end to a solid matrix,
15   a further modification of labeled glycosaminoglycan was
needed. This modification involved the placement of a
primary amino group at one end of the labeled
glycosaminoglycan. A preferable method of accomplishing
this placement was to incubate the labeled
20   glycosaminoglycan with an ammonium salt and sodium
cyanoborohydride at an alkaline pH. A Schiff base
initially forms between ammonia and the aldehydic
carbonyl group of the terminal hexose. This Schiff base
is reduced to a primary amine by the sodium
25   cyanoborohydride.

        The synthetic steps to produce the solid phase
substrate of the present invention generally include
partial N-deacylating, for example, by hydrazinolysis, or
30   N-desulfating, for example by solvolysis in dimethyl-
sulfoxide; an N-acylating step with labeled acyl
anhydride or halide for radioisotopic labeling; a
reductive amination step; and coupling to a solid matrix
through the newly introduced terminal amine. An often
35   preferred final step, to insure that no amine-reactive
functions remain on the solid matrix, is to incubate the

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product of the matrix -labeled amine-terminal glycos-
aminoglycan coupling with sodium cyanoborohydride and a
compound bearing a free amino group. This latter
compound may, for example, be one such as ethanolamine or
5   glycine ethyl ester.

The substrates and procedures of the present
invention present numerous advantages for the assay of
glycosaminoglycan endoglycosidase enzymic activity. For
10   example, the substrate of the present invention is bound
to a solid matrix via a single carbohydrate-bound amino
ligand and yields a linear pattern of enzymatic products.

In the past, proteoglycans containing glycosamino-
15   glycans as well as a bound protein component have been
bound to a solid matrix of cyanogen bromide-activated
agarose. The proteoglycan was thereby likely bound to
the agarose primarily through its proteinaceous com-
ponent. Thus, both proteolytic as well as glycosamino-
20   glycan endoglycosidic activity may liberate a soluble
product. The specificity of the assay for enzymic
activity of the endoglycosidases is less than the results
shown with the present invention.

25   A heparanase (heparan sulfate endoglycosidase)
obtained from a human melanoma cell line was found to
only partially degrade N-desulfated, N-acetylated
heparin. This same enzyme preparation was found to
efficiently cleave N-desulfated heparan sulfate as well
30   as N-desulfated N-acetylated heparan sulfate into
characteristic degradation fragments.

While there are many glycosaminoglycan endoglycosi-
dases, heparan sulfate endoglycosidase or heparanase, the
35   endoglycosidase utilizing heparan sulfate as a preferred
substrate, was chosen as a typical example to demonstrate

a preferred embodiment of the present invention. Addi-
tionally, an N-hydroxy succinide agarose derivative was
selected as a preferred solid matrix to couple labeled
amine-terminal heparan sulfate to produce a solid phase
5   substrate. Heparanase activity produced soluble
radioisotopically labeled products as demonstrated
specifically in many of the following examples.

Melanoma heparanase is an endo-β-glucuronidase which
10   specifically cleaves HS at intrachain sites. Such
melanoma heparanase specificity is illustrated in Figure
8. Thus, the separation of the reaction products from
the substrates based on their size is required for the
heparanase assay. Although previously established
15   methods such as polyacrylamide gel electrophoresis and
high-speed gel permeation chromatography are useful for
the characterization of degradation fragments, they are
not suitable for rapid and microscale quantitative assays
of large sample numbers. To perform rapid quantitative
20   assays, a covalently linked substrate is required. The
presently developed solid phase assay substrate is
partially N-deacetylated or N-desulfated, N-[^{3}H or ^{14}C]-
acetylated HS coupled with Affi-Gel 15. In this
substrate a HS derivative is linked to agarose through
25   only one covalent bond (Figure 1). This product is one
of the most sensitive endoglycosidase substrates to be
developed. This substrate has now been successfully used
for mouse and human melanoma heparanase assays. The same
type of derivative has also been produced by using
30   Reacti-Gel (NW-65F) (Pierce, Rockford, IL). However,
both Affi-Gel 15 and Reacti-Gel (HW-65F) use quite large
particles and these retain significant amounts of high
molecular weight materials in the gel matrices. This may
be a problem in some quantitative heparanase assays,
35   therefore, we developed a more desirable assay substrate
by using Affi-Gel 701 or 702 (Bio-Rad) which are

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approximately 1-3 microns in diameter with an exclusion
limit of M_r 10,000. The specific synthetic procedure was
as follows. Radiolabeled HS was reduced with sodium
borohydride to form a sugar alcohol at the reducing
5   terminal. The sugar alcohol was converted to a primary
aldehyde by periodate oxidization. This aldehyde group
was then linked to amino-derivatized beads, such as Affi-
Gel 701, through a Schiff base and stabilized by
reduction with sodium cyanoborohydride. Yet further
10   proposed procedures, similar to our previously developed
methods, are contemplated as useful. Radiolabeled HS
whose amino groups have been sulfated or acetylated
should be aminated at the reducing terminal with ammonia
under reducing conditions. Affi-Gel 702 should be
15   converted to an amino-reactive bead by derivatization
with N-hydroxysuccinimide or N,N'-carbonyldiimidazole,
and then the aminated radioactive HS should be linked to
amino-reactive Affi-Gel 702. The substrate may be made
more radioactive by use of ^{125}I-labeled HS, although
20   iodination of HS with Bolton and Hunter Reagent may be
disadvantageous because of potential structural change.
On the other hand, the assay may also be improved by use
of fluorescein-labeled HS for routine clinical studies,
and fluorescein-labeled HS is suitable for a rapid
25   analysis of degradation fragments on HPLC equipped with a
flow fluorescence detector.

The assay measuring levels of a glycosaminoglycan
endoglycosidase such as heparan sulfate endoglycosidase
30   (heparanase) may also be performed in an immunoassay
format using polyclonal and/or monoclonal antibodies
raised to the endoglycosidase. Preferably, antibodies
with relatively low cross-reactivity to other endoglyco-
sidases, such as the platelet endoglycosidase described
35   by Oldberg, et al. (1980) Biochem., V 19, pp 5755-5762,
can be used. The antibodies may be used with a variety

of immunoassay techniques to measure the endoglycosidase
protein directly. The endoglycosidase may be measured by
either a radioimmunoassay described by Berson and Yalow
(1968) <u>Clin. Chem. Acta.</u>, V 22, p 51 or an immunoradio-

5   metric (IRMA) assay described by Miles, et al. (1976)
<u>Anal. Biochem.</u>, V 61, pp 209-224 using 125I-labeled
antigen or antibody. The endoglycosidase may also be
measured by an enzyme immunoassay that uses either a
competitive-binding assay or a "sandwich" assay analogous

10   to an IRMA and using alkaline phosphatase, horse radish
peroxidase, or any other enzyme coupled to an antibody or
to the endoglycosidase as reviewed by Wisdom (1976) <u>Clin.
Chem.</u>, V 22, pp 1243-1255.

15   The cellular localization of tumor invasion- and
metastasis-associated degradative enzymes has been
studied biochemically as well as immunochemically.
However, most of our current knowledge on this subject is
based on studies that utilized proteolytic enzymes, such

20   as cathepsin B and type IV collagenase. Sloane and her
colleagues (Rozhin et al., Cancer Res., 47, 6620-6628
(1987); Sloane et al., Proc. Natl. Acad. Sci., 83, 2483-
2487 (1986) reported that cathepsin B-like activity in
metastatic B16 melanoma cells was detected in plasma

25   membrane-associated fractions. Keren et al., Cancer
Res., 48, 1416-1421 (1988) reported that cathepsin-B like
activity was present in 2% butanol extracts containing
cell surface associated molecules of metastatic B16
melanoma and fibrosarcoma cells, and the plasma membrane-

30   associated cathepsin B activity appeared to correlate
with the metastatic potentials of the tumor cells,
including melanoma, rhabdomyosarcoma, and mammary
carcinoma cells. There is a good correlation between
type IV collagenase activities in the conditioned media

35   of tumor cells and their invasiveness and metastatic
potentials (Liotta et al., Nature (Lond.) 284, 67-68

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(1980); Nakajima *et al., Cancer Res.*, 47, 4869-4876
(1987). Nakajima *et al., Cancer Res.*, 49, 1698-1706
(1989) found a plasma membrane associated 64 kDa type IV
collagenase on rat mammary adenocarcinoma cell surfaces.

5   Interestingly, in an immunohistochemical study with the
rabbit V2 carcinoma, Graf *et al., Lab. Invest.*, 45, 587-
596 (1981) observed that cathepsin B was localized to
normal cells, including fibroblasts and leukocytes, at
the tumor invasion front, but it was not found on the

10   carcinoma cells. Using indirect immunofluorescence
methods, Woolley *et al.*, In: P. Strauli, A.J. Barret,
and A. Baici (eds), *Proteinases and tumor invasion,* pp
97, Raven Press, New York (1980) and In: L. S. Liotta
and I.R. Hart (eds), *The Tumor invasion and metastasis,*

15   pp 391, Martinus Nijhoff, the Hague (1982) studied the
localization of collagenase in human primary and
metastatic melanoma tissues. Three out of 5 primary
melanoma specimens and 6 of 15 metastasis specimens
examined had positive staining with the anti-collagenase

20   antibodies, but the results were too variable to draw any
conclusions. They found fluorescence in the connective
tissue surrounding melanoma nests in some cases and at
the junctions between tumor and normal tissues in others.
Data from previous observations of the present inventors

25   on heparanase activity in melanoma cells suggested that
heparanase is localized in the cytoplasm and at the cell
surface. Heparanase activity has been recovered from
intact melanoma cells and their homogenates (Nakajima *et
al., Science*, 220, 611-613 (1983), as well as from

30   their shedded plasma membrane vesicles. Other reports
have also suggested that heparanase activities are plasma
membrane-associated or secreted by malignant cells.
Heparanase activity has been observed in the serum-free
conditioned medium of metastatic ESb murine lymphoma

35   cells and also in intact, viable ESb cells incubated with
[^{35}S]SO$_4$-labeled subendothelial extracellular matrix.

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Vlodavsky *et al., Cancer Res.*, 43, 2704-2711 (1983) . In
another study, Savion *et al., J. Cell. Physiol.*, 118,
169-178 (1984) reported that murine activated T
lymphocytes and inflammatory peritoneal macrophages are
5   able to release heparanase and degrade heparan sulfate in
the subendothelial extracellular matrix. These results
suggest cell surface association or cell secretion of
heparanase. On the other, Ricoveri and Cappelletti
Cancer Res., 46, 3855-3861 (1986) suggested that the
10   metastatic tumor cell heparanase is a lysosomal enzyme
and scarcely released in vitro. However, the
immunochemical localization of heparanase has not
previously been attempted.

15   Melanoma heparanase, an endo-β-D-glucuronidase, is
thought to play an important role in melanoma metastasis
(Nakajima *et al., J. Cell. Biochem.*, 36, 157-167 (1988).
It is one of several tumor cell degradative enzymes that
have been systematically studied. Heparanase activities
20   have been found in human and mouse melanoma cell
homogenates and their conditioned media as described
above. The successful preparation of anti-heparanase
enabled the study of the localization of murine and human
heparanases in normal and tumor tissues. To ensure the
25   specificity of the antibodies, extensive characterization
was carried out. Immunoprecipitates of the antibodies
with melanoma cell lysates were subjected to SDS-PAGE to
determine the molecular weights of the proteins the
antibodies recognized. The pIs of the immunoprecipitated
30   proteins were also examined using 2-dimensional gels. In
an attempt to aid the purification of heparanase, we also
constructed anti-heparanase antibody-agarose affinity
column using AffiGel 10 (Bio-Rad). Heparanase activity
was only detected in the fractions eluted with citrate
35   buffer at pH lower than 4. The finding that anti-murine
heparanase antibodies cross-react with human heparanase

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is consistent with our previous observations on human and
mouse heparanase. Human and mouse heparanases share a
number of biochemical characteristics, such as molecular
weight, substrate and inhibitor specificity, and optimal
5   pH for enzymatic activity. These observations suggest
that heparanase molecules may be highly conserved between
mouse and human.

The generation of anti-heparanase antibodies also
10 gave us an opportunity to localize heparanase in melanoma
cells and tissues. Heparanase antigen was localized
predominantly in the cytoplasm of both B16-BL6 and A375-
SM cells, as well as at the cell surface of these cells.
It remains to be determined which fraction of heparanase
15 antigen (cell surface, cytoplasmic or both) represents
the active form of the enzyme. The relationship between
cell surface, cytoplasmic, and secreted forms of
heparanase, and the regulatory mechanisms involved are
not clear. The immunocytochemical staining of B16-BL6
20 and A375-SM melanoma cells appeared to be heterogeneous,
which is consistent with the notion of tumor cell
heterogeneity in the expression of gene products.

Since heparanase activity has been correlated with
25 the metastatic potentials of murine B16 melanoma cell
lines (Nakajima et al., J. Biol. Chem., 259, 2283-2290
(1984), heparanase antigen was examined in mouse and
human melanoma tissues. Metastatic lung nodules in a
murine experimental metastatic model were intensely
30 stained with the anti-heparanase antibodies, although the
background staining in normal pulmonary tissues
surrounding the tumor was barely noticeable. The
background in normal lung tissue may be due to the
presence of the pulmonary alveolar macrophages in which
35 heparanase activity was observed (Nakajima et al., J.
Cell. Biol., 101, 215a (1985); Savion et al., J. Cell.

Physiol., **118**, 169-178 (1984). The present invention
takes advantage of the fact that the anti-mouse-
heparanase antibodies cross-reacted with human heparanase
and allowed study of the immunochemical localization of
5   heparanase antigen in human melanoma tissue. Melanoma
tissues from patients with metastatic diseases were
examined. Melanoma cells became stained with the
antibody but the surrounding normal tissues were not
except that the endothelial cells of an arteriole (Fig.
10   3c) were slightly stained. It is possible that
endothelial cells may express heparanase along with other
degradative enzymes in the process of neo-angiogenesis
and wound healing. The data presented in accord with the
present invention suggest that heparanase antigens in
15   invasive melanoma cells are significantly enriched
compared to surrounding normal tissues.

The localization of heparanase antigen at the
melanoma cell surfaces supports the concept of cell
20   surface involvement in tumor invasion and metastasis
(Nicolson, *Biochim. Biophys. Acta,* **695**, 113-176 (1982);
Moscatelli *et al., Biochim. Biophys. Acta,* **948**, 67-85
(1988). The localization of heparanase to invasive and
metastatic melanoma cells in melanoma tissues strongly
25   suggests that heparanase plays an important role in
melanoma invasion and metastasis. Such localization may
lead to changes in therapeutic strategy, more
metastatically or invasion prone tumors being likely to
contain more detectable heparanase and being worthy of
30   more aggressive therapy, for example.

The endoglycosidase may also be measured in these
assays by using fluorescein or other fluorescent
compounds as reviewed by Gerson (1984) J. Clin.
35   Immunoassay, V 7, pp 73-81, by chemiluminescence as
reviewed by Weeks and Woodhead (1984), J. Clin

Immunoassay, V 7, pp 82-89, or by other labels. In all
of these assays, the bound endoglycosidase may be
separated from the unbound endoglycosidase by a variety
of techniques.   These include solid-phase immobilization
5   of a primary (anti-endoglycosidase) antibody, avidin-
biotin separation using a biotin-labeled antibody and
solid phase avidin, "double antibody" precipitation, or
by using solid phase antibody against a hapten like
fluorescein coupled to a primary antibody, or by using a
10   solid phase "second antibody".   ("Double antibody" is
defined as a heterologous antibody that binds the anti-
endoglycosidase antibody as in Midley, et al. (1969) _Acta_
Endocrinol., V 63, Supp. 142, p 247).

15   The solid phase systems mentioned above can include
polymers, such as polystyrene; agarose; sepharose;
cellulose; glass beads; and magnetizable particles of
cellulose or other polymers.   The solid-phase can be in
the form of large or small beads or particles; tubes;
20   plates; or other forms.

Kits useful in the present invention include those
of the general type described by Szczesniak (U.S. Pat.
No. 3,899,298).   Such kits comprise a carrier being
25   compartmentalized to receive at least one, or at least
two or at least three or more containers and to maintain
said containers in closed confinement.   A first container
may contain purified anti glycosaminoglycan
endoglycosidase antibody (preferably monoclonal), either
30   in solution, in freeze-dried form or covalently bound to
the inside thereof, such as for example if such container
is a test tube.   A second container may then contain a
second anti glycosaminoglycan endoglycosidase antibody
(also preferably monoclonal).   Alternatively, another
35   container may contain detectably labeled
glycosaminoglycan endoglycosidase antigen.   At the time

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of testing for glycosaminoglycan endoglycosidase antigen
in the sample, the sample is added to the first container
containing the monoclonal antibody, incubated, and then
antibody from the second container is added thereto to
5 provide a "sandwich". The antibody in the second
container may be detectably labeled as, for example, by a
radiolabel or an enzyme label. Another container in the
kit may contain appropriate enzyme substrate in order to
carry out the "ELISA" methodology. Any number of
10 variations or permutations consistent with the various
techniques for use in the detection of glycosaminoglycan
endoglycosidase antigen may be envisioned for the
preparation of a kit. These are all matters of choice,
determined by the ease of handling, rapidity and
15 efficiency of the testing.

Quantitative analysis of glycosaminoglycan
endoglycosidase antigen can be carried out by
interpolation into a standard curve, as is known in the
20 art. A multiplicity of container means, each one having
a different amount of glycosaminoglycan endoglycosidase
antigen can be present in the kit for such a purpose.

In still another embodiment, the antibody can be
25 immobilized onto plastic strips which are then brought
into contact with the samples suspected of containing
glycosaminoglycan endoglycosidase antigen. Subsequently,
the strip is contacted with a solution containing a
second, enzyme labeled anti glycosaminoglycan endoglyco-
30 sidase antibody; this results in a sandwich forming on
the strip. Finally, introduction of the strip into a
color developing solution (such as substrate for the
enzyme) and detection of color, is a rapid efficient and
inexpensive method for qualitatively, and even roughly
35 quantitatively determining glycosaminoglycan
endoglycosidase antigen in animal samples.

The immunoassays of the present invention may use antibodies which are very discriminating between the different glycosaminoglycan endoglycosidases,

5   particularly for heparan sulfate endoglycosidase. The methodology described herein should be superior in sensitivity and ease to other known methods of glycosaminoglycan endoglycosidase detection.

10   In an analogous manner, kits are easily constructed comprising labeled glycosaminoglycan affixed, preferably through its amino-terminal end to a molecular tag as described above. Such a kit would also comprise a specific binding agent capable of removing tagged

15   glycosaminoglycan or tagged fragments thereof from solution. The specific binding agent may already be bound to a solid matrix or may be so bound by the user of the kit and assay. Preferred binding agents are proteins, more preferably, antibodies and most

20   preferably, monoclonal antibodies.

The development of specific diagnostic tests for infections of glycosaminoglycan endoglycosidase has become medically desirable for purposes such as detection

25   of tumors. Such specific diagnostic tests as described herein may be developed through the use of monoclonal or polyclonal antibodies specifically binding to glycosaminoglycan endoglycosidase.

30   These examples are presented to describe preferred embodiments and utilities of the present invention and are not meant to limit the present invention unless otherwise specified in the claims appended hereto.

35   <u>Purification of heparanase</u>
Melanoma heparanase was purified from cultured

murine melanoma cells. Murine B16-F10 melanoma cells
(Fidler, *Nature (London) New Biol.* 242, 148-149 (1973)
were cultured in a 1:1 mixture of Dulbecco's modified
Eagle's medium and Ham's F-12 nutrient mixture (DME/F12;
5   Hazleton, Lenexa, KS) supplemented with 5% heat-
inactivated fetal bovine serum (FBS; Hyclone, Logan, UT).
Cells in a subconfluent culture were harvested by a brief
treatment with 2 mM EDTA in phosphate buffered saline at
pH 7.2 (PBS) and 2×10^8 cells were extracted at 4°C for
10   30 min in 50 ml of 50 mM Tris-HCl, pH 7.5 containing 1 mM
phenylmethylsulfonyl fluoride (PMSF; sigma, St. Louis,
MO), 5 mM N-ethylmaleimide (NEM; Sigma), 0.5% Triton X-
100 and 0.05% sodium azide (buffer 1). The cell extract
was centrifuged at 30,000 x g for 30 min at 4°C and the
15   supernatant containing approximately 50 mg protein was
passed through a column of heparin-Sepharose (Pharmacia
LKB Biotechnology, Piscataway, NJ) equilibrated with
buffer 1. The heparin-Sepharose column was sequentially
washed with buffer 1, 20 mM sodium acetate, pH 6.0,
20   containing 0.2% Triton X-100 (buffer 2), and 0.15 M
sodium chloride, 20 mM sodium acetate, pH 6.0 (buffer 3).
Heparin-binding proteins were eluted with a linear sodium
chloride gradient (0.15 M - 1.2 M) in 20 mM sodium
acetate, pH 6.0. The eluted materials were monitored by
25   measuring absorbance at 280 nm, and the heparanase
activity was measured using heparanse solid phase
substrates as previously described (Nakajima et al. *Anal.
Biochem.*, 157, 162-171 (1986b). Heparanase active
fractions contained approximately 6 mg proteins and 90%
30   of total activity. After dialysis against buffer 3 the
heparanase fraction was centrifuged at 30,000 x g for 30
min and the supernatant was loaded on a concanavalin A-
Sepharose column (Pharmacia LKB) equilibrated with buffer
3. After washing the column with buffer 3, concanavalin
35   A-binding proteins were eluted with 1.0 M α-methyl-D-
mannopyranoside (Sigma) in buffer 3. The eluent

containing approximately 0.2 mg protein and 48% of total
heparanase activity was collected and extensively
dialyzed against buffer 3, and then passed through a N-
acetylated N-desulfated heparin-Sepharose column

5   (Nakajima et al. *Anl. Biochem.*, **157**, 162-171 (1986b)
equilibrated with the same buffer. After washing with 0.3
M sodium chloride, 20 mM sodium acetate, pH 6.0, binding
proteins were eluted with 0.6 M sodium chloride 20 mM
sodium acetate, pH 6.0. Highly purified heparanase

10 fractions (0.08 mg protein, 34% total activity) were
dialyzed against 12.5 mM Tris-HCl, 0.15 M NaCl, pH 7.5,
and concentrated with Centricon 30 concentrators (Amicon,
Danvers, MA), and then subjected to high speed gel
permeation chromatography using a Waters 600E system

15 equipped with a PROTEINPAK 300 SW column (Waters,
Milford, MA). The proteins were eluted with 12.5 mM
Tris-HCl, 0.15 M NaCl, pH 7.5 (1 ml/min, 23°C) and the
elution was monitored at 280 nm with a Waters Model 490
multiple wavelength detector. The second peak fractions

20 of molecular weight range approximately 100,000 to
150,000 contained heparanase (0.04 mg protein, 29% of
total activity). The heparanase fraction was further
subjected to chromatofocusing using a PBE94 column
(Pharmacia LKB). Starting buffer and elution buffer were

25 25 mM imidazole-HCl, pH 7.5 and Polybuffer 74-HCl
(Pharmacia LKB), pH 4.0, respectively. Heparanase was
eluted as a sharp peak at pH 5.0-5.2, and approximately
0.01 mg protein with 18% of total activity was recovered.
The purified heparanase was analyzed by sodium dodecyl

30 sulfate (SDS)-polyacrylamide gel electrophoresis
according to the methods by Laemmli *Nature (London.)* **227**,
680-685, (1970) and its apparent molecular weight was
determined as 97,000. The heparanase band was cut and
electroeluted using an ISCO electrophoretic concentrator

35 Model 1750 (ISCO, Lincoln, NE) and its N-terminal amino
acid sequence was analyzed by a model 470A gas-phase

sequencer (Applied Biosystem Inc.) with an attached model
120A-PTH analyzer.

Immunological reagents

5   Heparanase peptide (EEDLGKSREGSRTDD-C) was designed
based on an analysis of the hydrophilicity of amino acid
residues of the heparanase N-terminal sequence
(EVDVDGTVEEDLLGKSREGSRTDD) and was synthesized on a
DuPont solid phase peptide synthesizer according to the
10   method by Merrifield *J. Am. Chem. Soc.*, **85**, 2149-2154
(1963). The peptide was designed to have an additional
cysteine residue at C-terminus to facilitate coupling to
a carrier protein, keyhole limpet hemocyanin (KLH;
Calbiochem, La Jolla, CA). Other protein carriers are of
15   course usable in place of KLH. Coupling of the peptides
with KLH was performed by the methods described by Liu et
al., *Biochemistry,* **18**, 690-697 (1979). Briefly, LKH was
suspended in 10 mM sodium phosphate buffer pH 7.2 (PB) at
20 mg/ml and dialyzed using a cellulose membrane with a
20   molecular weight cutoff of 12,000-14,000 (Spectrum, Los
Angeles, CA) against 2 L of the same buffer overnight (3
changes). The dialysate was centrifuged at 10,000 x g
for 10 min to remove undissolved material. The
concentration of KLH was adjusted to 16 mg/ml after
25   protein concentration was determined by the Pierce
Coomassie protein assay (Pierce, Rockford, IL) using
bovine serum albumin (Sigma) as a standard. The peptide
was dissolved in PB at a concentration of 5 mg/ml, and m-
maleimidobenzoyl-N-hydroxysuccinimide ester (MBS; Sigma)
30   was dissolved in dimethylformamide (DMF; Aldrich,
Milwaukee, WI) at t concentration of 6 mg/ml. The MBS
solution was slowly added to the LKH solution (MBS:KLH =
0.51 mg:4.0 mg) and the mixture incubated at 25°C for 30
min with gentle stirring. The activated KLH was
35   separated from remaining low molecular weight MBS by gel
filtration chromatography. It appeared at the void

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volume of a Bio-Gel P-30 gel filtration column (2.5 x 25
cm) and was eluted with 50 mM phosphate buffer, pH 6.0.
The peptide solution (5 mg/ml) was then mixed with the
activated KLH, and the pH was adjusted with sodium

5   hydroxide to 7.0-7.5. After incubation at 25°C for 3 h,
the peptide-KLH mixture was centrifuged at 10,000 x g to
remove undissolved material. The coupling efficiency was
determined by the Ellman assay for free thiol (Ellman et
al., *Arch. Biochem. Biophys.*, **82**, 70-77 (1959). The

10   coupling efficiency was greater than 78.5%. The KLH-
coupled peptide antigens were aliquoted and stored at -
80°C.

New Zealand White rabbits were immunized

15   subcutaneously with KLH-coupled heparanase-peptide.
Prior to injection, the antigen was emulsified with
either complete freund's adjuvant for the primary
immunization or incomplete Freund's adjuvant for subse-
quent boosting immunizations. Dosage of antigen was 500

20   ug of peptide for the first injection and 250 µg for
subsequent boosting injections. The interval between
first and second injections was 3 weeks and boosting
injections were administered when the antisera titer
began to drop. Antibody activity to heparanase-peptide

25   was determined using an enzyme-linked immunosorbant assay
with peptide coated 96-well plates (Costar, Cambridge,
MA). The plates were prepared by coating each well with
1 µg of synthetic peptide antigen in 100 µl of 0.1 M
sodium bicarbonate, pH 9.0, and allowing the buffer to

30   evaporate at 37°C overnight. The titers of antisera
raised against heparanase peptides were between 1:800 and
1:1200 when preimmune sera were used as reference.

The antibodies directed against heparanase-peptide

35   were further purified by antigen affinity chromatography
using heparanase-peptide covalently coupled to Affi-Gel

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10 beads (Bio-Rad, Richmond, CA). The sera were first
precipitated with 45% (v/v) ammonium sulfate at 4°C and
centrifuged at 10,000 x g for 20 min. The precipitates
were then dialyzed against 1000 vol of 5 mM HEPES, 150 mM

5   NaCl, pH 7.4, overnight at 4°C with 3 changes of buffer.
The dialysates were loaded on Affi-Gel-heparanase-peptide
affinity columns. The affinity columns were washed
extensively with 5 mM HEPES, 150 mM NaCl, pH 7.4, and
then eluted with 50 mM sodium citrate, 150 mM NaCl, pH

10 5.5, and finally with 50 mM sodium citrate, 150 mM NaCl,
pH 2.0. The fractions were collected and protein
concentrations determined by the Pierce Coomassie Protein
Assay, using IgG as the protein standard. Antibody
activity to heparanase-peptide was determined by ELISA

15 assay. Antibody fractions eluted at pH 2.0 were
collected and designated as anti-heparanase antibodies.
The antibodies were biotinylated according to the
procedure of Updyke et al., *J. Immunol. Meth.*, **73**, 83-95
(1984).

20

The heparanase-peptide was alkylated with
iodoacetamide to inactivate the sulfydryl group at the C-
terminus of the peptide and used to compete with anti-
heparanase antibodies as a control in an

25 immunoprecipitation experiment. The reaction was carried
out at a concentration of 2 mM peptide, 1 mM
dithiothreitol, 20 mM iodoacetamide, in 50 mM Tris, 150
mM sodium chloride, pH 7.5, at 25°C for 1 hr. The sample
was concentrated and the alkylated peptide was separated

30 on a Bio-Gel P-2 column (mesh 400). The void volume was
collected and lyophilized.

Cells and cell culture

Human A375-MM and A375-SM melanoma cell lines

35 (Kozlowski et al., *J. Natl. Cancer Inst.*, **72**, 913-917
(1984), which were selected in nude mice for increased

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lung colonizing potential, were a gift of Dr. I.J. Fidler
(University of Texas M.D. Anderson Cancer Center,
Houston, TX) Murine B16-BL6 melanoma cells, sequentially
selected for increased bladder invasion (Hart, *Am. J.*
5   *Pathol.*, **97**, 587-600 (1979), were from Dr. I.R. Hart
(Imperial Cancer Research Laboratories, London, England).
Cells were cultured in 1:1 (v/v) mixture of DME/F12
supplemented with 5% heat inactivated FBS in a tissue
culture incubator gassed with 5% CO_2 at 37°C. Cells were
10   grown in multichamber slides (Miles Scientific,
Naperville, IL) for immunocytochemical studies.

Immunoprecipitation and Autoradiography
          Melanoma cells were plated onto 10-cm tissue culture
15   dishes. At subconfluency, the medium was aspirated and
the cells were gently rinsed with Dulbecco's phosphate
buffered saline (DPBS) once and then supplemented with
DME/F12 plus 5% dialyzed FBS in the presence of 50 μCi/ml
of [^{35}S]methionine (ICN, Irvine, CA). After 24 hr of
20   metabolic labeling, the cells were rinsed with DPBS and
incubated for 10 min with 2 mM EDTA in Ca^{2+},Mg^{2+}-free PBS.
The cells were then centrifuged at 600 x g for 5 min, the
pellet lysed with 0.5% Triton X-100, 50 mM Tris-HCl, 1 mM
PMSF, 5 mM NEM pH 7.5, at a ratio of 1 ml per 6 x 10^6
25   cells, and the incubation continued at 25°C for 10 min
with mixing on a Vortex machine. The specific activity
was then determined by measuring trichloroacetic acid-
precipitable radioactivity, and the protein
concentration of the cell lysate was determined. The
30   specific activity was higher than 5 x 10^6 cpm/μg of
protein. A cell lysate containing 200 μg of anti-
heparanase antibodies for 1 hr at 4°C, followed by
incubation with 10 μl of Streptavidin-Agarose (BRL,
Bethesda, MD) for 1 hr at 4°C with mixing on a Vortex
35   machine. The samples were washed once with 10 mM HEPES,
150 mM NaCl, pH 7.5, containing 0.5% Triton X-100,

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followed by washing with 10 mM HEPES, 150 mM NaCl, pH
7.5, 4 times. The precipitates were suspended in SDS
sample buffer in the presence or absence of 2.5% ß-
mercaptoethanol and heated at 100°C for 3 min. They were
5   then electrophoresed on a 7.5% polyacrylamide gel (Bio-
Rad, Richmond, CA) in the presence of SDS. The gel was
then rinsed briefly with deionized water, dried, and
placed with Kodak XOMAT film on an intensify screen for 2
days at -80°C prior to developing. ^{14}C-labeled molecular
10   weight standards (Sigma) were included in the
electrophoresis.

Mouse and human melanoma tissues
        Mouse metastatic melanoma tissues were prepared as
15   follows. Subconfluent B16-BL6 cell monolayers were
rinsed with DPBS and incubated with 2 mM EDTA in Ca^{2+},
Mg^{2+}-free PBS for 10 min, suspended as single cells in
DME/F12, chilled in an ice-water bath before
centrifugation, and resuspended in cold DME/F12 at a
20   concentration of 2.5×10^5 cells/ml. Just prior to
injection, the cell suspension was warmed to room
temperature, and cell aliquots (5×10^4 cells in 0.20 ml)
were injected into the lateral tail vein of 6- to 8-week-
old C57/BL6 mice using a 27-gauge needle. Ten mice were
25   used in each experiment. After 2-3 weeks, the mice were
exsanguinated under anesthesia and the lungs were
carefully taken for frozen sectioning.

        Human melanoma tissues were obtained from seven
30   patients diagnosed with malignant melanoma. Human nevi
biopsies were contributed by normal donors. Frozen
tissue sections were prepared as described below.

Indirect immunocytochemistry and immunohistochemistry
35   Melanoma cells grown on multichamber slides (Miles
Scientific) were fixed at 25°C for 20 min with 2%

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paraformaldehyde in PBS, and then rinsed three times with
PBS. Some cells were permeabilized with cold acetone
at -20°C for 3-10 min and rinsed 3 times with PBS at
25°C. The fresh mouse and human tissues were mounted on
5   stubs with O.C.T. mounting medium (Miles Scientific) in
the vapor phase of liquid nitrogen and subsequently
sectioned (2 μm thickness) on a cryostat microtome
(Damon/IEC custom microtome, Needham Heights, MA).
Sections were fixed with 2% paraformaldehyde in PBS at
10   25°C for 20 min. After 3 rinses with PBS, the slides
were incubated for 1 hr with preimmune rabbit IgG and
then with PBS, anti-heparanase antibodies, or anti-
heparanase antibodies pre-incubated with synthetic
peptide antigens for 1 hr at 25°C. Following 3 rinses
15   with PBS, the slides were incubated with peroxidase-
conjugated goat anti-rabbit IgG (Calbiochem, La Jolla,
CA) at a 1:150 dilution in PBS or PBS for 1 hr. The
slides were then developed in 3-amino-9-ethylcarbazole
(AEC; Sigma) buffer: 4 mg of AEC dissolved in 1.0 ml of
20   N,N-dimethylformamide (Aldrich) in 100 ml 0.1 M sodium
acetate, pH 5.2, plus of 1-2 drops 30% H_2O_2 for 20 min.
The slides were covered with cover slips using glycerol-
gelatin mounting medium prepared by dissolving 10 g of
gelatin (Fisher Scientific) in 60 ml distilled water with
25   heating until the gelatin dissolved, and supplementing
the gelatin solution with 70 ml glycerin and 1 ml phenol.

Glycans and enzymes. Bovine lung heparan sulfate
was (HS) a kind gift from Dr. N. Di Ferrante (Baylor
30   College of Medicine, Houston, TX) and its average M_r was
determined as 34,000 by sedimentation equilibrium
(Nakajima, M., et al., (1984) J. Biol. Chem. V 259, pp
2283-2290 and Irimura, T., et al., (1983) Anal. Biochem.
V 130, pp 461-468). Heparin (M_r 11,000) from porcine
35   mucosal tissue was kindly donated by Drs. M. B. Mathews,
J. A. Cifonelli, and L. Roden (University of Chicago,

IL). Chondroitin 6-sulfate (C6S) from shark cartilage
was obtained from Miles Scientific (Naperville, IL) and
further purified by gel chromatography; its average M_r was
determined as 60,000 as described previously (Irimura,
5   T., et al., (1983) Anal. Biochem. V 130, pp 461-468).
Heparin from bovine lung and porcine intestinal mucosa
and N-acetyl-D-glucosamine were obtained from Sigma
Chemical Co. (St. Louis, MO). Monosialosyl biantennary
complex-type glycopeptide UB-I-b (M_r 2190) was prepared
10 from thyroglobulin (Sigma) (Irimura, T., et al., (1983)
Anal. Biochem. V 130, pp 461-468). Heparitinase from
Flavobacterium heparinum (EC4.2.2.8) was obtained from
Miles Scientific.

15   High-speed gel permeation chromatography. High-
speed gel permeation chromatography was carried out using
a high pressure liquid chromatograph system (LDC, Riviera
Beach, FL) equipped with two sequential columns (0.7 x 75
cm) of Fractogel (Toyopearl) TSK HW-55(S) (MCB,
20 Gibbstown, NJ) as described previously (Irimura, T., et
al., (1983) Anal. Biochem. V 130, pp 461-468). A one
hundred microliter aliquot of sample solution was
delivered into the injection port, and the
chromatographic elution was performed with 0.2 M sodium
25 chloride at a flow rate of 1.0 ml/min at 55°C (Irimura,
T., et al., (1983) Anal. Biochem. V 130, pp 461-468). In
the analysis of radiolabeled materials, fractions
corresponding to each 36 s of elution (0.6 ml) were
collected and mixed with 3.0 ml of Liquiscint (National
30 Diagnostics, Comerville, NJ), and counted on a Beckman LS
2800 liquid scintillation counter (Beckman Instruments,
Irvine, CA).

     Cellulose acetate electrophoresis. Glycosamino-
35 glycans were analyzed by cellulose acetate electro-
phoresis according to the method of Cappelletti et al.

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(Cappelletti, et al., Anal. Biochem., V 99, pp 311-315).

Titan III Zip Zone cellulose acetate plates (6.0 x
7.6 cm, Helena Laboratories, Beaumont, TX) were used, and
5   electrophoresis was carried out at 70 V for 60 min in 0.5
M pyridine-acetate (pH 5.0), instead of 0.1 M barium
acetate buffer employed by Cappelletti et al.
(Cappelletti, et al., Anal. Biochem., V 99, pp 311-315).
During the electrophoresis the buffer and cellulose
10   acetate plates were kept below 4°C using petroleum ether
cooled with ice.

N-Desulfation and acetylation of HS. N-desulfation
of HS was conducted by the methods of Nagasawa and Inoue
15   (Nagasawa et al., (1977) Methods in Carbohyd. Chem. V 8,
pp 291-294). The sodium salt of purified HS was
converted to the pyridinium salt by cation exchange
chromatography on a column of AG50WX8(H$^+$ form, Bio-Rad,
Richmond, CA) and neutralization with pyridine. Complete
20   N-desulfation and partial N-desulfation of HS was carried
out by solvolysis of the pyridinium salt of HS in 95%
dimethylsulfoxide (DMSO) and 5% water for 120 min at
50°C, and for 60 min at 20°C, respectively. The pH of
the reaction mixture was adjusted to 9.0 by the addition
25   of 0.1 M sodium hydroxide; and then the mixture was
dialyzed against running tap water overnight and then
against distilled water for 20 h. The N-acetylation of
N-desulfated HS was performed in 4 M sodium acetate, pH
8.0, containing 4% acetic anhydride, 15% methanol for 3 h
30   at 4°C. The reaction mixture was dialyzed against
running tap water overnight and then against distilled
water, and the mixture was then lyophilized.

Radioisotope labeling of HS. To study the effects
35   of chemical modification of HS on its susceptibility to
melanoma heparanase, HS was labeled with tritium at the

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reducing end as described previously (Nakajima, M., et
al., (1984) J. Biol. Chem. V 259, pp 2283-2290). One
milligram of purified HS was reduced with 2 mCi of
NaB[^{3}H]$_4$(340 mCi/mmol; New England Nuclear, Boston, MA) in

5   0.1 M sodium borate buffer, pH 8.0, for 5 h at 25°C.
After acidification to pH 5 with acetic acid, the
reaction mixture was chromatographed on a column (1.0 x
105 cm) of Sephacryl S-200 equilibrated with 0.2 M
pyridine-acetate buffer, pH 5.0. Fractions of ^{3}H-labeled

10   HS with specific M_r were collected and lyophilized. To
synthesize radiolabeled HS for a solid-phase heparanase
substrate, partially N-desulfated HS was N-acetylated
with [1-^{14}C]acetic anhydride. Fifteen milligrams of
partially N-desulfated HS were incubated with 0.15 mCi of

15   [1-^{14}C]acetic anhydride (10.0 mCi/mmol; New England
Nuclear) in 4 M sodium acetate, pH 8.0, for 4 h at 4°C;
and then further incubated with 4% acetic anhydride in
the same buffer for 4 h at 4°C. The reaction mixture was
extensively dialyzed against distilled water, and then

20   lyophilized. High M_r fractions of partially N-desulfated
N-[^{14}C]acetylated HS (PNDS-N[^{14}C]Ac-HS) were obtained by
gel chromatography on a column of Sephacryl S-200 as
described above.

25   <u>Reductive Amination and Coupling of ^{14}C-labeled HS to</u>
<u>amino-reactive agarose beads</u>. The reducing terminal
saccharides of ^{14}C-labeled HS were reductively aminated as
follows. PNDS-N[^{14}C]Ac-HS (5 mg) was dissolved in 5 ml of
distilled water and mixed with 5 ml of 4 M ammonium

30   formate and 0.8 M sodium cyanoborohydride in methanol,
and then incubated at 50°C for 7 days. The reaction
mixture was dialyzed against distilled water and
lyophilized. The reductively aminated products were
dissolved in 10 ml of 0.1 N sodium bicarbonate, pH 8.5,

35   and mixed with Affi-Gel 15 (or Affi-Gel 10; Bio-Rad)
prepared from the original suspension by successive

washing in 2-propanol and then ice-cold distilled water.
The mixture was incubated at 4°C for 24 h with gentle
mixing. The pH was then adjusted to pH 8.5 with 0.1 N
sodium bicarbonate and the incubation further continued.

5   After 24 h the unreacted sites on the Affi-Gel 15 were
blocked by addition of 1 ml of 1 M glycine ethyl ester
(pH 8.0), and the beads were again incubated for 6 h at
4°C. After the reaction was complete, the coupling
products were extensively washed in 1.5 M sodium

10   chloride, and incubated in 0.1 M sodium acetate, 0.15 M
sodium chloride, 0.2% Triton X-100, and 0.05% sodium
azide (pH 6.0) at 37°C overnight. The products were
further washed in the same buffer and stored at 4°C.

15   The summary of the above described synthetic
procedures is shown in Figure 1.

     Chemical deacetylation and radioactive reacetylation
of heparan sulfate and its coupling to agarose beads.

20   ^{14}C-or ^{3}H-labeled HS were prepared by chemical deacetyla-
tion and radioactive reacetylation as follows. Nine
milligrams of bovine lung HS (provided by Dr. N. Di
Ferrante, Baylor College of Medicine, Houston, Tx.) were
dried with 1 mg hydrazine sulfate over phosphorous

25   pentoxide under vacuum at 50°C for 48 hrs. Anhydrous
hydrazine (0.5 mg, Pierce Chemical, Rockford, Il.) was
added to the dried HS, and the mixture was heated in a
tightly screwed tube under nitrogen atmosphere at 100°C
for 1 hr. After the reaction, the hydrazine was removed

30   by repeated evaporation with toluene over sulfuric acid
dessicant under vacuum conditions. To separate deacety-
lated HS from residual reagents and partial degradation
products, completely dried residue was dissolved in 0.5
ml water and subjected to gel filtration on a 0.8 x 30 cm

35   column of BioGel p-10 (400 mesh) eluting with distilled
water. The void volume fraction was collected and

lyophilized. The yield was approximately 60% by weight.
The N-deacetylated HS was then N-acetylated with 50 uCi
[^{14}C]-acetic anhydride (10 mCi/mmole: NEN, Boston, Ma.)
or 5 mCi ^{3}H-acetic anhydride (400 mCi/mmole:NEN) in 0.5 ml

5   of 4 M sodium acetate for 18 hrs. Complete N-Acetylation
was accomplished by mixing with 0.1 ml of non-labeled
acetic anhydride for 1 hr. ^{14}C- or ^{3}H-labeled HS was
purified on the same BioGel P-10 column as described
above.

10

For the solid-phase heparanase assay, ^{3}H-HS was
aminated at the reducing terminal with 2 M ammonium
acetate in the presence of 0.4 M sodium cyanoborohydride
in 50% methanol at 50°C for 6 days. Aminated ^{3}H-HS was

15   purified by gel filtration as above, and the resulting
solution was made to 0.1 M in sodium carbonate. To 10^{6}
cpm of aminated ^{3}H-HS, 1.0 ml Affi-Gel 15 was added after
the gel beads were washed with isopropanol and chilled
water according to the manufacturer's recommendations.

20   The coupling reaction was continued at 4°C for 48 hrs
with continuous agitation. The agarose beads were then
washed with 4 M sodium chloride repeatedly to remove non-
covalently attached ^{3}H-HS from the beads.

25   <u>Melanoma cells and cell culture</u>. The high lung-
colonizing metastatic murine B16 melanoma subline (B16-
F10) and fourteen established cell lines of human
malignant melanoma were employed in this study. The
human melanoma cell lines used were: SK-MEL-19, SK-MEL-

30   23, SK-MEL-93(DX1), SK-MEL-93(DX3), SK-MEL-93(DX6),
Hs294T, Hs852T, HS939, T294, M40, RON, BMCL, A375 parent,
and A375 Met Mix. A375 Met Mix cells were prepared from
spontaneous lung metastases of A375 parental cells in the
athymic nude mice and both A375 cell lines were provided

35   by Dr. I.J. Fidler (The University of Texas-M.D. Anderson
Hospital and Tumor Institute, Houston, TX). Melanoma

cells were grown on plastic tissue culture dishes in a
1:1 mixture of Dulbecco's modified minimum essential
medium and Ham's F12 medium (DMEM/F12; Gibco
Laboratories, Grand Island, NY) with 10% fetal bovine
5   serum (Hyclone, Sterile Systems, Inc., Logan, UT) and
without antibiotics, under humidified conditions in a 95%
air-5% CO_2 atmosphere. All cell cultures were determined
to be free of mycoplasma contamination with the use of
mycoplasma detection system (BRL MycoTest; Bethesda
10   Research Laboratories, Gaithersburg, MD).

        Preparation of cell extracts. Subconfluent melanoma
cells were harvested by treatment for 10 min with 2 mM
ethylene diamine tetracetic acid (EDTA) in Ca^{2+}, Mg^{2+}-free
15   DPBS. Single cell suspensions were washed twice by brief
centrifugation in 0.14 M sodium chloride, 10 mM Tris-HCl
buffer, pH 7.5, and checked for viability (usually >95%)
by trypan blue dye exclusion. Cells were suspended in
chilled 50 mM Tris-HCl buffer, pH 7.5, containing 0.5%
20   Triton X-100 at a concentration of 6×10^6 cells/ml and
extracted for 5 ml at 25°C and for an additional 1 h at
4°C. The supernatant was collected after centrifugation
at 9800 x g for 5 min at 4°C. Protein contents in the
centrifugated extracts were determined by a modification
25   of the Lowry technique to correct for the presence of
Triton X-100 in the samples (Nakajima, M., et al., (1984)
J. Biol. Chem. V 259, pp 2283-2290).

        Enzymatic degradation of unmodified and modified HS.
30   In the enzymatic degradation experiments the ^{3}H-labeled HS
substrate (10 ug) was incubated with a B16-F10 cell
extract (80 ug protein) in 200 ul of 0.1 M sodium acetate
buffer (pH 6.0) containing 0.15 M sodium chloride, 0.2%
Triton X-100 and 0.05% sodium azide (reaction buffer A)
35   in the presence of 20 mM D-saccharic acid 1,4-lactone
(SAL, a potent exo-β-glucuronidase inhibitor). The

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incubation was carried out at 37°C with gentle mixing,
and was terminated by chilling the mixture to 4°C and
adding 20 ul of trichloroacetic acid to a final
concentration of 5%. The supernatant was obtained by
5   centrifugation at 9800 x g for 5 min and it was subjected
to analysis by high-speed gel permeation chromatography.

Heparanase assay using a solid-phase substrate. A
suspension of PNDS-N[^{14}C]Ac-HS coupled to Affi-Gel 15 was
10   mixed with a melanoma cell extract and incubated in 400
ul of reaction buffer A containing 20 mM SAL. The enzyme
reaction was terminated by chilling the solution to 4°C
and mixing it with 40 ul of 50% trichloroacetic acid.
After incubation for 10 min at 4°C, the mixture was
15   centrifuged at 9800 x g for 5 min, and the supernatant
was withdrawn. Two hundred microliter aliquots of the
supernatant were mixed with 55 ul of 1.0 N sodium
hydroxide and 4 ml of Liquiscint (National Diagnostic)
and counted in a Beckman LS 2800 liquid scintillation
20   counter.

Effects of N-desulfation and N-acetylation of HS on
HS degradation by melanoma heparanase. To label purified
HS with radioactive molecules without use of linking
25   reagents that might cause significant changes in HS
molecular structure, we replaced some of the N-sulfate
groups with N-[^{14}C]acetyl groups. This idea was based on
our previous observation that B16 melanoma heparanase was
highly active against various HS molecules but inactive
30   against heparin, and that HS differs from heparin in its
high N-acetyl and low N-sulfate contents.

The effect of N-desulfation and N-acetylation of HS
on its susceptibility to melanoma heparanase was assessed
35   using HS labeled with tritium at the reducing terminal
saccharide. Since HS purified from bovine lung had

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mostly N-acetyl-D-glucosamine at the reducing ends
(Nakajima, M., et al., (1984) J. Biol. Chem. V 259, pp
2283-2290), HS was reduced with NaB[³H]₄. HS labeled with
tritium at the reducing end was N-desulfated by

5   solvolysis with DMSO, and N-desulfated [³H]HS was then N-
acetylated with acetic anhydride. These three ³H-labeled,
chemically modified HS molecules were analyzed by
cellulose acetate electrophoresis in 0.5 M pyridine-
acetate buffer, pH 5.0. The relative mobilities of HS N-

10   desulfated HS (NDS-HS) and N-desulfated N-acetylated HS
(NDS-NAc-HS) under the electrophoresis conditions
described in the materials and methods were 3.30, 2.55,
and 2.90, respectively. These findings indicated that N-
desulfation of HS resulted in a significant loss of

15   negative charge; however, the total negative charge was
partially recovered by acetylation of free amino groups.
The average molecular size of NDS-HS and NDS-NAc-HS were
determined by high-speed gel permeation chromatography,
and were found to be 30,000 and 31,500, respectively

20   (Fig. 2). Each of HS, NDS-HS and NDS and NDS-NAc-HS was
incubated with B16 melanoma cell extracts in the presence
of SAL (a potent exo-β-glucuronidase inhibitor), and the
incubation products were analyzed by high-speed gel
permeation chromatography. All these substrates were

25   cleaved by melanoma heparanase at high rates and the
elution profiles of their degradation products were
similar, although the M_r of the fragments produced were
characteristic for each substrate (Fig. 2). Degradation
of each of the chemically modified HS was totally

30   inhibited by addition of amounts of heparin purified from
bovine lung or porcine intestinal mucosa (data not
shown). The results indicated that N-sulfate in HS may
not be important for its recognition and cleavage by
melanoma heparanase, and that the chemical modification

35   of sulfated amino groups in HS does not significantly
affect its degradation by heparanase.

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N-Desulfated and N-acetylated heparin. The known
structures of HS and heparin suggested that N-desulfation
and subsequent N-acetylation of heparin may generate
5   local structures similar to those present in HS. Heparin
is a potent inhibitor of B16 melanoma heparanase
(Nakajima, M., et al., (1984) J. Biol. Chem. V 259, pp
2283-2290); however, its heparanase inhibitory activity
is lost by the removal of N-sulfate (Irimura, et al.
10   (1985) J. Cell. Biochem. V 9A, p 148). Since the results
above suggested that N-sulfate in HS is unnecessary for
its cleavage by melanoma heparanase, N-desulfated N-
acetylated heparin was used as a heparanase substrate.
Heparin from porcine intestinal mucosa (M_r 11,000)
15   previously labeled with tritium at its reducing end
(Nakajima, M., et al., (1984) J. Biol. Chem. V 259, pp
2283-2290) was N-desulfated and then N-acetylated by the
procedures employed in the preparation of NDS-NAc-HS.
The product, N-desulfated N-acetylated heparin (NDS-NAc-
20   heparin), had an apparent M_r of about 10,500 as determined
by high-speed gel permeation chromatography; and its
relative electrophoretic mobility on cellulose acetate in
0.2 M pyridine-acetate buffer, pH 5.0, was 0.87 when the
electrophoretic mobility of ^{3}H-labeled heparin was taken
25   as 1.00. ^{3}H-labeled heparin and ^{3}H-labeled NDS-NAc-
heparin were incubated with B16 cell extracts and the
reaction products were analyzed by high-speed gel permea-
tion chromatography. Percentage degradation of the
original substrates was calculated from the decrease in
30   area of the high M_r half of the substrate peak as reported
previously (Nakajima, M., et al., (1984) J. Biol. Chem. V
259, pp 2283-2290). During the first 6 h incubation with
a B16 cell extract, less than 5% of heparin was degraded,
while approximately 20% of the NDS-NAc-heparin was
35   fragmented. However, NDS-NAc-heparin was not further
cleaved and the major peak of NDS-NAc-heparin on high-

speed gel permeation chromatography did not shift to a
lower M$_r$ even after a prolonged incubation. This
suggested that N-desulfation and subsequent N-acetylation
of heparin can result in the generation and/or exposure
5   of heparanase-susceptible glucuronosyl linkages in a part
of the heparin molecule. Thus, NDS-NAc-heparin cannot be
utilized as a melanoma heparanase assay substrate.

     <u>Synthesis of solid-phase substrate for melanoma</u>
10   <u>heparanase assay</u>. The procedure for the synthesis of a
solid-phase substrate for heparanase is illustrated in
Fig. 1. To minimize the radiolabeling effects on the HS
structure, HS was only partially N-desulfated by
solvolysis in 95% DMSO and 5% H$_2$O for 1 h at 20°C.
15   Partially N-desulfated HS was acetylated with [1-
^{14}C]acetic anhydride as described in the material and
methods section. The remaining free amino groups were
completely acetylated with acetic anhydride. These steps
yielded PNDS-N[^{14}C]Ac-HS, (M^r 33,000) with radioactivity
20   of 294 cpm/ug. The relative mobility of PNDS-N[^{14}C]Ac-HS
on cellulose acetate electrophoresis was 3.15, indicating
that the total negative charge of PNDS-N[^{14}C]Ac-HS is much
closer to that of unmodified HS than is that of NDS-NAc-
HS as expected.
25

     The reducing terminal saccharides of PNDS-N[^{14}C]Ac-HS
were reductively aminated with 2M ammonium formate and
0.4 M sodium cyanoborohydride in 50% methanol. The
products having free amino groups only on the reducing
30   termini were then coupled to aminoreactive agarose beads
such as Affi-Gel 10 or Affi-Gel 15. Incubation of 5 mg
of PNDS-N[^{14}C]Ac-HS with 0.5 ml of packed Affil-Gel 15
resulted in the immobilization of 10.2% PNDS-N[^{14}C]Ac-HS
onto Affi-Gel 15 (0.51 mg PNDS-N[^{14}C]Ac-HS per 0.5 ml of
35   gel). Increasing concentrations of PNDS-N[^{14}C]Ac-HS up to
10 mg per 0.5 ml of Affi-Gel 15 did not significantly

affect the coupling efficiency under the conditions used.

PNDS-N[^{14}C]Ac-HS was also conjugated to Affi-Gel 10
under the same conditions used for the coupling of PNDS-
5   N[^{14}C]Ac-HS to Affi-Gel 15. However, the coupling
efficiency was low, less than 1%, between pH 7.5 and 8.5.
Therefore, a positive charge spacer at the aminoreactive
site of Affi-Gel 15 may be important in the effective
coupling of PNDS-N[^{14}C]Ac-HS to Affi-Gels. Using Affi-Gel
10   15, one of the best heparanase assay substrates was
produced: PNDS-N[^{14}C]Ac-HS immobilized on agarose through
only one covalent linkage at the reducing terminal end.

     Enzymatic degradation of PNDS-N[^{14}C]Ac-HS immobilized
15   on agarose gel beads. The susceptibility of PNDS-
N[^{14}C]Ac-HS immobilized on agarose gel to HS degrading
enzymes was examined by incubating the substrates (4500
cpm, 15 ug) with bacterial heparitinase (EC.4.2.2.8) at a
concentration of 5 units/ml in 0.1 M sodium acetate
20   buffer, pH 7.0, containing 1 mM calcium acetate (Linker,
et al. (1972) Methods Enzymol. V 28, pp 902-911). Most
of ^{14}C activity (82%) appeared in the supernatant of the
incubation mixture after a 24 h incubation, indicating
that PNDS-N[^{14}C]Ac-HS immobilized on agarose is very
25   susceptible to HS degrading enzymes. The remainder of
the PNDS-N[^{14}C]Ac-HS was not released from the gel, even
after prolonged incubation. This could be explained by
the limitation of using a Flavobacterium heparitinase.
The same amount of substrate (4500 cpm) was incubated
30   with B16 cell extract for various periods in the presence
of 20 mM SAL to prevent the sequential degradation by
exogylcosidases. The relationships between the amounts
of cell extract (ug protein) added and the release ^{14}C
activity are shown in Fig. 3. In this case, the maximum
35   amount of released ^{14}C activity were 56% of the total ^{14}C
activity present in the solid-phase substrates. A

portion of the ^{14}C activity could not be released by
melanoma heparanase, since the incubation of HS or of
chemically modified HS with a B16 cell extract resulted
in the production of large fragments with the original

5   reducing termini (Fig. 2). A linear relationship between
the amount of cell extract added and the release ^{14}C
activity was found for each incubation period (Fig. 3).
Since the results from the 12 h incubation were linear
over the widest range of cell extract amounts, we

10 measured the degradation of PNDS-n[^{14}C]Ac-HS during a 12 h
incubation.

The effect of heparin on the degradation of the
solid-phase substrates was investigated by addition of

15 substrate-equal amount (15 ug) of heparin from porcine
intestinal mucosa or heparin from bovine lung to the
incubation mixture containing B16-F10 cell extract (80 ug
protein) and PNDS-N[^{14}C]Ac-HS immobilized on agarose. The
addition of either heparin caused up to 80% inhibition of

20 the degradation of the solid-phase substrates, consistent
with our previous results (Nakajima, M., et al., (1984)
J. Biol. Chem. V 259, pp 2283-2290).

       <u>Measurement of heparanase activity in human melanoma</u>

25 <u>cells by use of PNDS-N[^{14}C]Ac-HS immobilized on agarose</u>
<u>beads</u>. Using PNDS-N[^{14}C]Ac-H immobilized on agarose
beads, the following fifteen human melanoma cell lines
were tested for heparanase activity: SK-MEL-19, SK-MEL-
23, SK-MEL-93(DX1), SK-MEL-93(DX3), SK-MEL-93(DX6),

30 Hs: 4T, Hs852T, Hs939, T294, M40, RON, BMCL, A375 parent,
A375 Met Mix, and A375 M6. All the human melanoma cells
showed the ability to degrade HS in the presence of SAL
as shown in Table 1. Six of these human malignant
melanoma cell lines such as SK-MEL-93(DX1), SK-MEL-

35 93(DX6), Hs939, M40, A375 Met Mix, and A375 M6
demonstrated significantly greater ability to degrade HS

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than did mouse B16 melanoma subline F10.

Interestingly, A375 Met Mix and A375 M6 cells were
selected from A375 parental cells by their ability to
5   colonize the lung in athymic nude mice. They were
reported to have a high metastatic potential, while A375
parental cells had a very low metastatic potential
(Kozlowski, et al. (1984) J. Natl. Cancer Inst. V 72, pp
913-917). Therefore, the heparanase activity of A375
10   cells may correlate with their spontaneous lung
metastatic potential.

The present inventors have previously found that
intact B16 melanoma cells or B16 cell extracts from
15   sublines of high lung colonization potential degrade
purified HS at higher rates than B16 cells of poor lung
colonization potential (Nakajima, et al. (1983) Science V
220, pp 611-613), and that B16 melanoma HS degrading
endoglycosidase is an endo-β-glucuronidase (heparanase)
20   (Nakajima, M., et al., (1984) J. Biol. Chem. V 259, pp
2283-2290).

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TABLE 1

HEPARAN SULFATE DEGRADATION ACTIVITY
IN HUMAN MALIGNANT MELANOMA CELLS

Melanoma cell lines ·	Heparan sulfate degradation activity[a] mean ± S.D. (cpm)
SK-MEL-19	379±40
SK-MEL-23	397±29
SK-MEL-93(DX1)	625±36
SK-MEL-93(DX3)	381±25
SK-MEL-93(DX6)	703±19
Hs294T	381±44
Hs852T	202±16
Hs939	619±44
T294	366±15
M40 ·	787±75
RON	457±27
BMCL	118±31
A375 parent[b]	392±38
A375 Met Mix[b]	659±22
A375 M6[b]	612±48
B16-F10 (mouse melanoma)	510±34

[a]Heparanase assay was carried out by the incubation of a Triton X-100 cell extract (2.4×10^5 cells) with PNDS-N[^{14}C]Ac-HS immobilized on agarose beads (4500 cpm) at 37°C for 12 h. The details of experiment are described in the materials and methods section. The radioactivity released in the presence of heat inactivated enzymes was subtracted from the raw data.

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^bA375 Met Mix and A375 M6 cells derived from lung
metastases of A375 parental cells in athymic nude mice
possess highly spontaneous lung metastatic potential,
while A375 parental cells have very low spontaneous
5   metastatic potential.

Heparanase Activity in Sera from Tumor Bearing Hosts

       Preparation of blood sera. Blood was withdrawn by
10   venepuncture without anticoagulant and allowed to clot
       for 1 hr at 22°C. Samples were centrifuged at 4°C for
       10 min at 800 x g and for 15 min at 1600 x g. The
       resultant sera were divided into small aliquots and
       snap-frozen in liquid nitrogen, and then maintained
15   at -80°C until analyzed.

       Assay of sera for heparanase. The serum was
       diluted 5-fold with 0.1 M sodium acetate buffer, pH
       6.0, containing 0.15M sodium chloride. Two hundred
20   microliter aliquot of the diluted serum was mixed with
       200 ul of radiolabeled solid phase substrate suspension
       (3,000 cpm, 10 ug) in the same buffer and incubated at
       37°C. At various incubation periods the enzyme
       reaction was terminated by chilling to 4°C, and the
25   reaction sample was mixed with 40 ul of 50%
       trichloroacetic acid. The mixture was incubated for 10
       min at 4°C and centrifuged at 9800 x g for 5 min. A
       two hundred microliter aliquot of the supernatant was
       withdrawn, neutralized with 55 ul of 1.0 N sodium
30   hydroxide, and then mixed with 4 ml of Liquiscint
       (National Diagnostic). The radioactivity was measured
       by a Beckman LS 2800 liquid scintillation counter.
       There was a linear relationship between incubation time
       and enzyme reaction. The activity was reported as
35   units per milliliter serum. One unit of activity

refers to the amounts of enzyme that liberates 1 ug of
HS per minute.

5   <u>Heparanase activity in the sera from malignant
melanoma patients</u>. The sera from 20 melanoma patients
at the various stages of the disease and from 15 normal
adults were assayed for heparanase, and the results are
shown in Fig. 4. The mean value and standard deviation
of heparanase activity in the sera from melanoma
10   patients and normal adults were 0.0177 ± 0.0075 and
0.0096 ± 0.0025 U/ml, respectively. The sera from some
patients who have been treated by chemotherapy showed
the normal levels of heparanase activity.

15   <u>Heparanase activity in the sera from rats bearing
13762NF mammary adenocarcinoma</u>. Highly metastatic
mammary adenocarcinoma MTLn3 cells (1×10^6) were
injected subcutaneously into the left inguinal mammary
fat pad of age matched female F344 rats. Rats were
20   sacrificed at various periods post-injection, and the
size of primary tumors, the number of lung metastases,
and the serum heparanase activity were measured. The
heparan sulfate degradative activities in sera
increased with time after the subcutaneous inoculation
25   of MTLn3 cells (Fig. 5). The activities in sera
correlated with the sizes of the primary tumors
(correlation coefficient $r = 0.770$, Fig. 6). The sera
from rats with large numbers of metastases in the lymph
nodes and lungs demonstrated much higher heparanase
30   activities than the sera from rats with few or no meta-
stasis (Fig. 7).

<u>Purification of melanoma heparanase</u>. Melanoma
cells (murine B16 melanoma subline B16-BL6 or human
35   melanoma Hs 939 cells) were grown in a 1:1 mixture of
DME/F12 medium supplemented with 5% heat-inactivated

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fetal bovine serum. Subconfluent cells were harvested
by a treatment for 10 min with 2 mM EDTA in PBS and
then washed twice in 0.14 M NaCl, 10 mM Tris-HCl
buffer, pH 7.2. The following steps were performed at
5   4°C. Cells (2 x 10^8) were extracted in 30 ml of 50 mM
Tris-HCl buffer, pH 7.2, containing 0.2% Triton X-100,
10 uM PMSF (buffer A) for 1 hr. The supernatant
(approximately 1.5 mg protein/ml) was collected after
centrifugation at 30,000 x g for 30 min, and was loaded
10   on a column of concanavalin A-Sepharose 4B (2 x 10 cm)
equilibrated with buffer A. After washing with 10 ml
of buffer A, the absorbed material was eluted with 1 M
alpha-methyl-D-mannoside in buffer A. The eluents were
filtered through a heparin-sepharose CL-6B column (2 x
15   10 cm) equilibrated with 50 mM Tris HCl buffer, pH 7.2,
containing 0.15 M sodium chloride 0.2% Triton X-100.
The column was washed with 100 ml of the same buffer.
and 100 ml of 0.15 M sodium chloride 50 mM Tris-HCl, pH
7.2, and then heparin-bound proteins were eluted with a
20   linear salt gradient (0.15 M-1.2 M sodium chloride).
The heparanase active fractions were collected and
dialyzed against 0.15 M sodium chloride and 0.01 M
potassium phosphate, pH 6.0. After centrifugation at
50,000 x g for 30 min, the supernatant was loaded on a
25   hydroxylapatite column (1.5 x 45 cm) equilibrated with
0.15 M sodium chloride and 0.01 M potassium phosphate,
pH 6.0. Heparanase was eluted with a linear gradient
of potassium phosphate (0.01 M to 0.6 M) in 0.15 M
sodium chloride, pH 6.0. The heparanase fractions were
30   concentrated by ultrafiltration using YM-10 membranes,
and were subjected to the further purification by
Sepharose CL-6B gel filtration. The Sepharose CL-6B
chromatography was performed in 0.15 M sodium chloride,
and 20 mM potassium phosphate, pH 6.0. Further
35   chromatography, now with Sephadex G-150, was carried
out in 0.5 M sodium chloride and 25 mM Tris-HCl, pH

7.5.   A single heparanase peak obtained from the
Sephadex chromatography contained a glycoprotein of M_r
96,000.

5   <u>Properties of human melanoma heparanase</u>.   Melanoma
heparanase is active between pH 5.5 and 7.5 and
degrades heparan sulfate but not other
glycosaminoglycans.   Heparin and dextransulfate are
potent inhibitors of melanoma heparanase.

10

TABLE 2

HEPARANASE SUSCEPTIBILITY AND HEPARANASE INHIBITORY ACTIVITY OF CHEMICALLY MODIFIED HEPARAN SULFATE AND HEPARIN

HS Glycosaminoglycans Degradation[2]	Inhibition of Degradation[1]
Heparan sulfate (HS)	+	-
N,O-Desulfated HS	+	-
N,O-Desulfated and N-acetylated HS	+	-
N-Desulfated HS	+	-
N-Desulfated and N-acetylated HS	+	-
Heparin	-	++
N,O-Desulfated heparin	-	-
N,O-Desulfated and N-acetylated heparin		±
N-Desulfated heparin	-	-
N-Desulfated and N-acetylated heparin	±	+
N-Desulfated and N-methylated heparin	-	-
Carboxyl reduced heparin	-	±

[1] ^{3}H-labeled glycosaminoglycan was incubated with a cell extract (80 ug of protein) in 0.1 M sodium acetate buffer (pH 6.0) containing 0.15 M NaCl, 0.2% Triton X-100 and 0.05% NaN$_3$ for 6 hr at 37°C in the presence of 20 mM D-saccharic acid 1,4-lactone (SAL) and was then subjected to high-speed gel-permeation chromatography.

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Percent of degradation was determined by measuring the
decrease in area of the high M_r half of the
glycosaminoglycan peak (see Figure 2). +, more than 80%;
±, 5% to 15%; -, less than 5% (S.D. < 5.0%).

5

[2] Five micrograms of unlabeled glycosaminoglycan was
added to the incubated mixture of ^{3}H-labeled HS from
bovine lung and a cell extract. Inhibition of HS
degradation was determined by measuring the decrease in

10 area of the high M_r half of the HS peak. ++, more than
80% inhibition; +, 25-50% inhibition; ±, 5-25%
inhibition; -, less than 5% inhibition (S.D. < 5%).

Human and Murine Heparanase Are Antigenically Related

15   Anti-heparanase antibodies directed against the
amino terminus of murine melanoma heparanase were
prepared as described above and some of the antibodies
were subsequently biotinylated. In an
immunoprecipitation experiment, human A375-SM (Fig. 9a-

20 d) and mouse B16-BL6 (Fig. 9e-h) melanoma cell lysates
metabolically labeled with [^{34}S]methionine were first
incubated with biotinylated anti-heparanase antibodies
(Fig. 9b,c,f,g) and then with streptavidin-agarose. The
antibody specifically immunoprecipitated a major

25 M_r~97,000 protein or a doublet of M_r~97,000 proteins
from mouse B16-BL6 (Fig. 9f,g) and human A375-SM (Fig.
9b,c) melanoma cell lysates, respectively. The protein
profiles on SDS-PAGE appeared to be the same under
nonreducing and reducing conditions.

30

          The specificity of anti-heparanase antibodies is
demonstrated in Fig. 9. The M_r~97,000 bands were not
noted in the presence of excess amount of competing
heparanase N-terminal peptide (Fig. 9a,e). Proteins of

35 M_r~205,000, ~125,000, ~76,000, ~52,000, and ~46,000
appeared to be precipitated nonspecifically or to be

related to streptavidin-agarose contaminants (Fig.
9d,h). In addition to these non-specific protein bands,
a faint band of $M_r \sim 105,000$ (Fig. 9b,c,e,f) appeared to
be related to anti-heparanase antibody precipitation but
5   its precipitation could not be inhibited by excess
amount of heparanase peptide ((Fig. 9a,e). Therefore,
it was considered to be a nonspecific component.

The antibodies immunoprecipitated a human $M_r \sim 97,000$
10   protein from A375SM cell lysates just as well as that
from B16-BL6 mouse melanoma cell lysates. Therefore,
the human and mouse heparanase molecules are similar in
molecular weight and antigenically related. To further
confirm the specificities of the antibodies against
15   heparanase, we examined the immunoprecipitates on 2-
dimensional gels. The pI of the immunoprecipitates were
the same as that of the purified heparanase: 5.0-5.2.

Heparanase Molecules are Immunochemically Localized at
20   the Melanoma Cell Surface and in the Cytoplasm

Acetone-permeabilized human A375-SM (Fig. 10a) and
mouse B16-BL6 melanoma cells were intensely stained with
the anti-heparanase antibodies in an indirect
25   immunochemical assay. The immunocytochemical staining
of A375-SM and B16-BL6 cells with anti-heparanase
antibodies appeared to be heterogeneous from cell to
cell (Fig. 10a, b). Nonpermeabilized A375SM (Fig. 10b)
and B16-BL6 cells, in which the antibodies can only
30   interact with antigens on the cell surface, were also
stained although not as intensely as their permeablized
counterparts. The B16-BL6 cells were stained
essentially the same as the A375SM cells; however, the

presence of melanin deposits sometimes interfered with
the immunoprecipitates. In contrast to the intense
staining of the melanoma cells by anti-heparanase
antibodies, there was no detectable immunoprecipitates.

5   In contrast to the intense staining of the melanoma
cells by anti-heparanase antibodies, there was no
detectable immunocytochemical staining in any of the
controls (Fig. 10c-f), including one where anti-
heparanase antibodies were preincubated with the N-

10 terminal heparanase peptide (Fig. 10d).

Mouse Melanoma Cells are Stained with Anti-heparanase
Antibodies in Experimental Metastatic Tumors

Using immunohistochemical techniques, heparanase was

15 localized in experimental mouse metastatic melanoma
tumors. Frozen sections of mouse lung tissue containing
micrometastatic melanoma nodules were specifically
stained with anti-heparanase antibodies. Examination
showed heparanase antigen localized in the tumor, not in

20 normal tissue (Fig. 11f). The sections presented in
Fig. 11d-f were taken from different levels of the same
micrometastatic tumor nodule. Serial sections show the
morphology of the micrometastatic lesion in the upper
right corner of the micrograph (Fig. 11d). The

25 micrometastatic melanoma nodule appeared to contain
large quantities of melanin, and the melanoma cells
appeared to be heterogeneous in size and have larger

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nuclei than the adjacent pulmonary alveolar epithelial

cells. The micrometastatic melanoma nodules were

intensely stained with anti-heparanase antibodies, and

the intensity of staining in the metastatic melanoma

5   tissue was higher than that in the adjacent lung tissue

(Fig. 11f). The same set of controls as those shown in

Fig. 2 was carried out, but only a representative

control is presented (Fig. 11e). No staining was found

in any of the controls, and the presence of melanin in

10   melanoma nodules was profound.

Metastatic and Invasive Melanoma Cells are Stained with

Anti-heparanase Antibodies in Human Metastatic Melanoma

Tissues

15   Human melanoma tissues from seven patients with

metastatic melanomas were examined for the presence of

heparanase using immunohistochemical techniques. The

melanoma cells were stained with anti-heparanase

antibodies, and in each case the surrounding tissues,

20   including connective tissue, were essentially unstained.

As a representative case, a cutaneous melanoma is

presented (Fig. 11a-c). The malignant melanoma cells,

which had invaded dermis connective tissue, are shown

adjacent to an arteriole and remnants of collagen fibers

25   (Fig. 11a). In this case, the tumor cells were

amelanotic and heterogeneous in size. Anti-heparanase

antibodies that stained melanoma cells did not stain

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surrounding normal parenchymal cells except slightly the
endothelial cells in a blood vessel (Fig. 11c). The
same controls as those shown in Fig. 10 were performed,
but only a representative one is presented (Fig. 11b).

5   In addition, human nevi were examined for the presence
of heparanase antigen using the same procedure as the
melanoma tissues. The melanocytes in the nevi and
adjacent normal skin were not stained by the anti-
heparanase antibodies.

10

    Changes may be made in the construction, operation
and arrangement of the various parts, elements, steps
and procedures described herein without departing from
the concept and scope of the invention as defined in the

15 following claims.

CLAIMS:

1.   An immunoassay method for detecting the presence of
a glycosaminoglycan endoglycosidase comprising
5   combining a human biological sample suspected of
containing glycosaminoglycan endoglycosidase with at
least one antibody specifically binding the
glycosaminoglycan endoglycosidase and determining
binding of said antibody to the sample as indicative of
10   glycosaminoglycan endoglycosidase amount and
localization.

2.   The method of claim 1 wherein the antibody is
15   polyclonal.

3.   The method of claim 1 wherein the antibody is
directed toward an N-terminal region of the
20   glycosaminoglycan endoglycosidase.

4.   The method of claim 1 wherein the immunoassay is a
radioimmunoassay, an enzyme immunoassay, or a
25   fluorescent immunoassay.

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5.   The method of claim 1 wherein the glycosaminoglycan
endoglycosidase is heparanase.

5   6.   An immunoassay method for detecting the presence of
a heparan sulfate endoglycosidase comprising combining
a buffered aqueous solution of a human biological
sample suspected of containing heparan sulfate
endoglycosidase with at lease one antibody raised to
10   the heparan sulfate endoglycosidase and determining the
level of binding of said antibody or antibodies to the
sample as indicative of the presence of heparan sulfate
endoglycosidase.

15

7.   The method of claim 6 wherein the antibody is
polyclonal.

20   8.   The method of claim 6 wherein the heparan sulfate
endoglycosidase is heparanase.

9.   The method of claim 6 wherein the immunoassay is a
25   radioimmunoassay, an enzyme immunoassay, or a
fluorescent immunoassay.

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10. A kit useful for the detection of a
glycosaminoglycan endoglycosidase in a sample which
comprises:

5   a carrier being compartmentalized to receive one or
                more container means in close confinement
                therein;

           a first container means comprising an antibody
10   which is specific for glycosaminoglycan
                endoglycosidase;

           a second container means comprising a detectably
                labeled antibody which specifically binds
15   glycosaminoglycan endoglycosidase.

11. A kit useful for the detection of glycosaminoglycan
endoglycosidase in a sample which comprises:

20

           a carrier being compartmentalized to receive one or
                more container means in close confinement
                therein;

25   a first container means comprising an antibody
                which specifically binds glycosaminoglycan
                endoglycosidase;

a second container means comprising detectably
labeled glycosaminoglycan endoglycosidase.

5

12. The kit of claim 10 or 11 wherein said antibody in
said first container means is immobilized on said
container means.

10

13. The kit of claim 10 wherein said antibody in said
second container is labeled with a radiolabel, an
enzyme label, biotin, a fluorescent label or a
chromophore.

15

14. The kit of claim 10 wherein the antibody in said
first container means or second container means is a
polyclonal antibody.

20

15. The kit of claim 10 or 11 which also comprises a
multiplicity of container means with different amounts
of glycosaminoglycan endoglycosidase antigen.

25

16.   The kit of claim 10 or 11 wherein said first
container means or second container means is a tube.

5   17.   The kit of claim 10 or 11 wherein the
glycosaminoglycan endoglycosidase is heparanase.

18.   A kit useful for the detection of a heparan sulfate
10   endoglycosidase in a sample which comprises:

            a carrier being compartmentalized to receive
            one or more container means in close
            confinement therein;
15

            a first container means comprising an antibody
            which specifically binds heparan sulfate
            endoglycosidase;

20   a second container means comprising a detectably
            labeled antibody specifically binding heparan
            sulfate endoglycosidase.

25   19.   A kit useful for the detection of heparan sulfate
endoglycosidase in a sample which comprises:

a carrier being compartmentalized to receive one or
more container means in close confinement therein;

5
            a first container means comprising an antibody
                which specifically binds heparan sulfate
                endoglycosidase;

            a second container means comprising detectably
                labeled heparan sulfate endoglycosidase
10   antigen.

20. The kit of claim 18 or 19 wherein said antibody in
said first container means is immobilized on said
15   container means.

21. The kit of claim 18 wherein said antibody in said
second container is labeled with a radiolabel, an
20   enzyme label, a fluorescent label or a chromophore.

22. The kit of claim 18 wherein said antibody in said
second container means is a monoclonal antibody.

25

23. The kit of claim 18 or 19 wherein the heparan

sulfate endoglycosidase is heparanase.

24.   The kit of claim 18 or 19 wherein the antibody in
5   the first container means is polyclonal.

25.   The kit of claim 18 or 19 which also comprises a
multiplicity of container means with different amounts
10   of heparan sulfate endoglycosidase antigen.

26.   The kit of claim 18 or 19 wherein said container
means is a tube.
15

27.   A peptide for immunogenically raising antibodies to
heparanase, the peptide consisting essentially of
EEDLGKSREGSRTDD.
20

28.   The peptide of claim 27 defined further as being
EEDLGKSREGSRTDDC.

25
29.   A method of raising antibodies specifically binding
heparanase, the method comprising immunizing an animal

with a carrier coupled to a peptide from the N-terminal
region of heparanase.

5   30.   The method of claim 29 wherein the peptide is that
of claim 27 or 28.

31.   The method of claim 29 wherein the carrier is a
10   protein.

32.   The method of claim 29 wherein the carrier is
keyhole limpet hemocyanin.

15

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N - DESULFATION WITH DMSO

N - ACETYLATION WITH ^{14}C-ACETIC ANHYDRIDE R = CH$_3$ ^{14}CO -

REDUCTIVE AMINATION WITH NaBH$_3$CN, HCOONH$_4$

AFFI-GEL 15 (OCH$_2$CONH(CH$_2$)$_3$N(CH$_2$)$_3$NHCO(CH$_2$)$_2$ CO

COUPLING TO AFFI-GEL 15
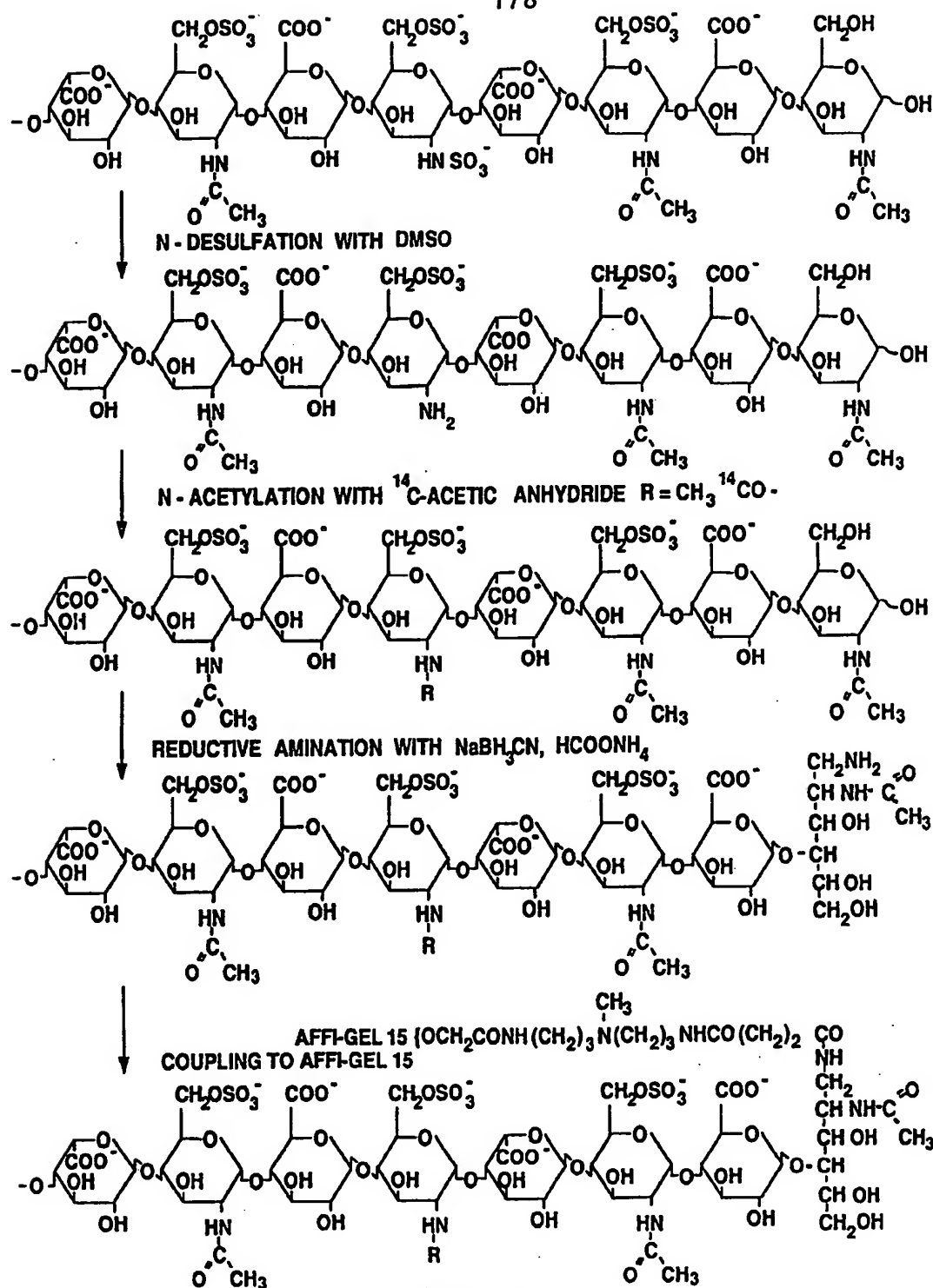
Fig. 1
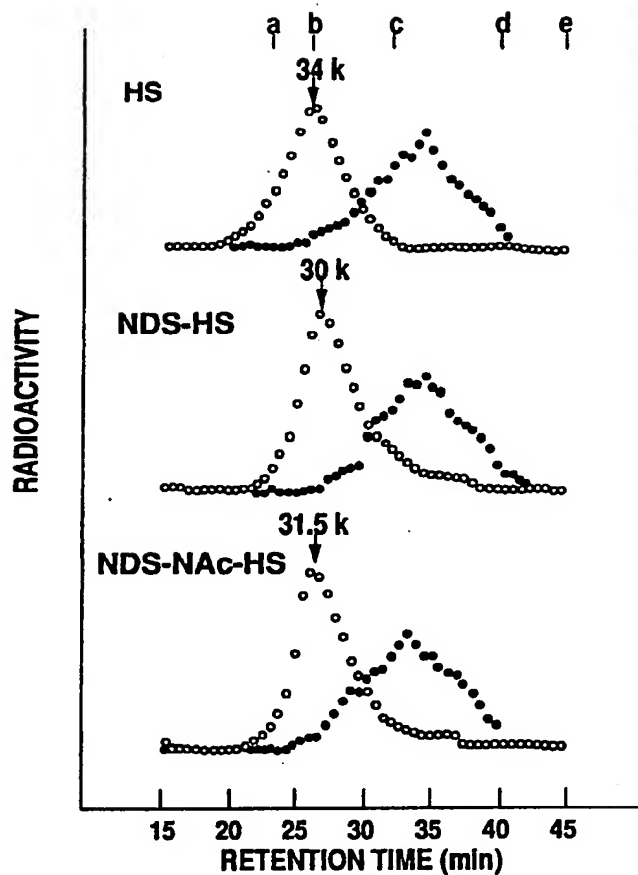
SUBSTITUTE SHEET

Fig. 2
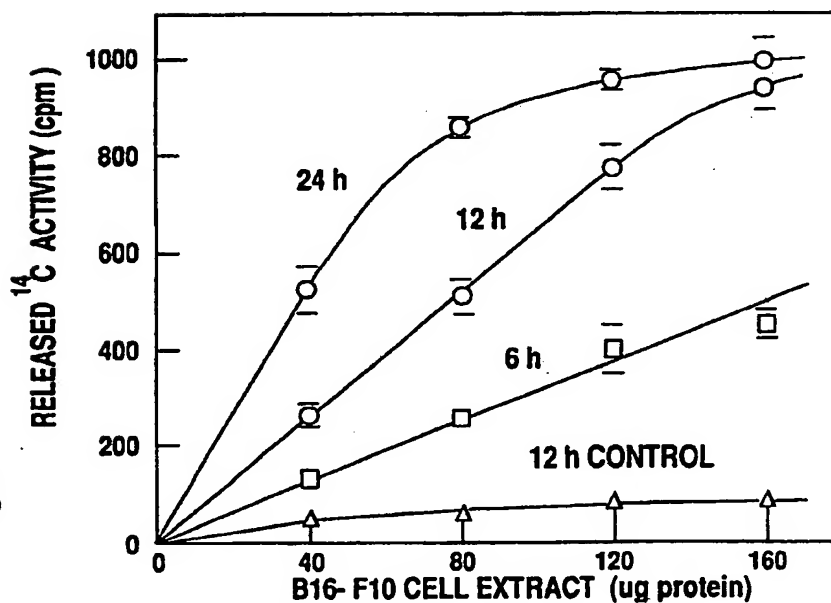
Fig.3

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Fig. 4

DAYS AFTER INOCULATION OF MTLn3 CELLS
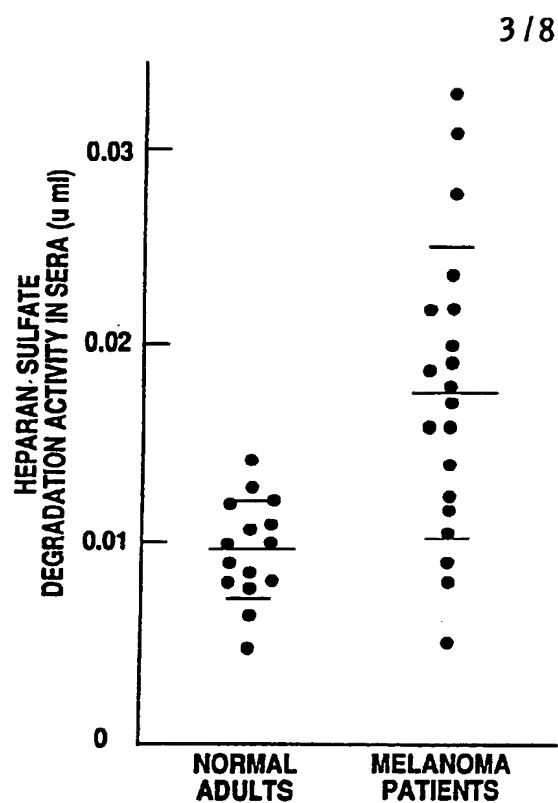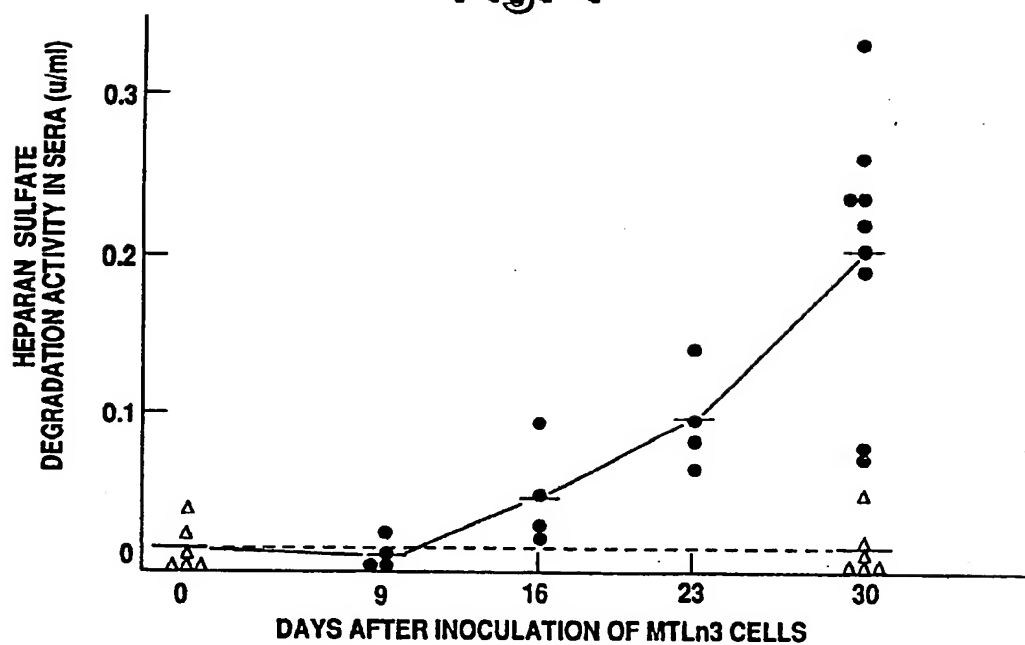
Fig. 5

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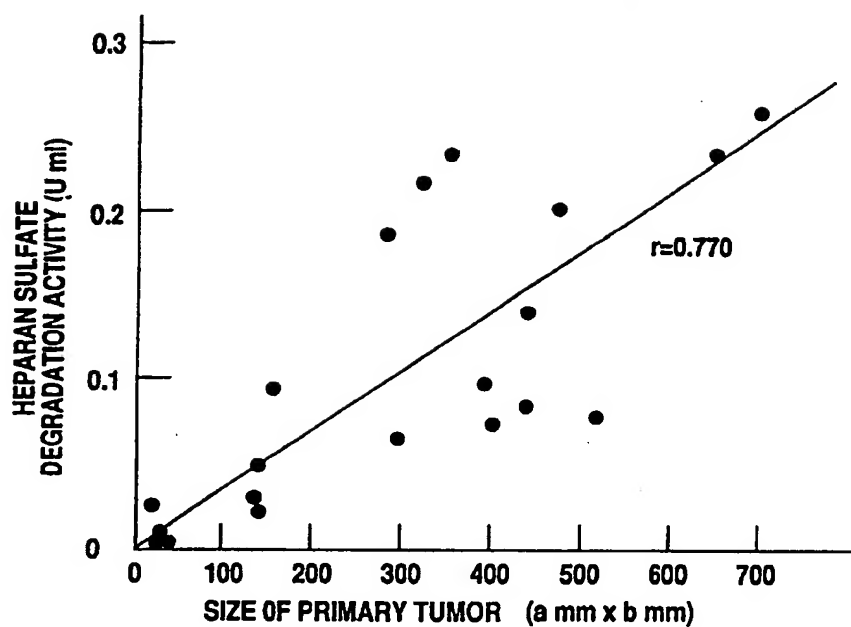
Fig.6
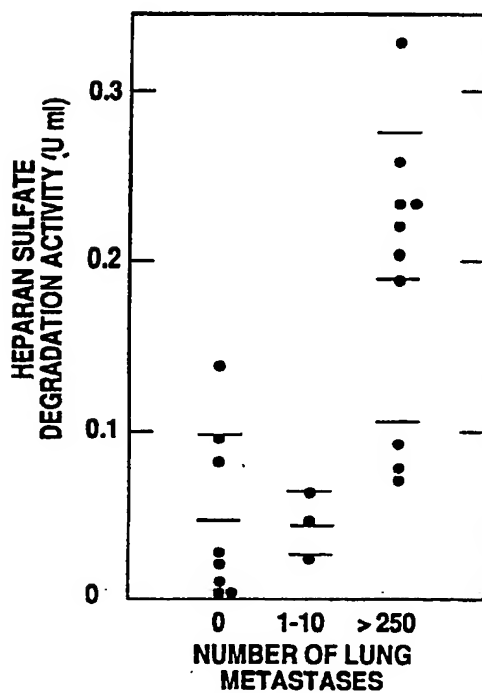
Fig.7a
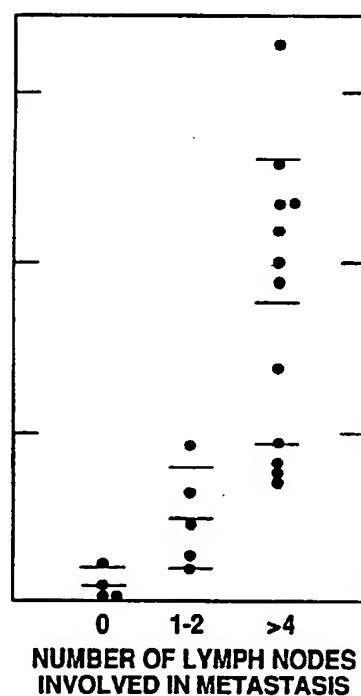
Fig.7b

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HEPARAN SULFATE PROTEOGLYCAN
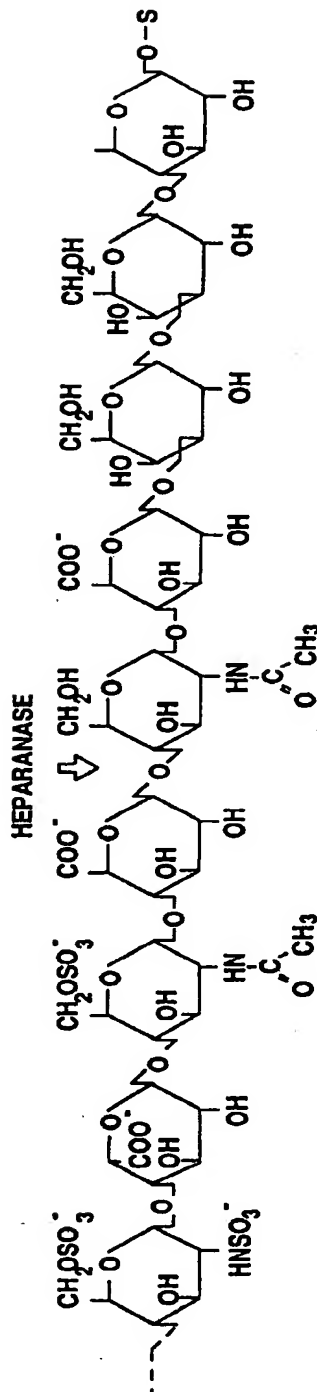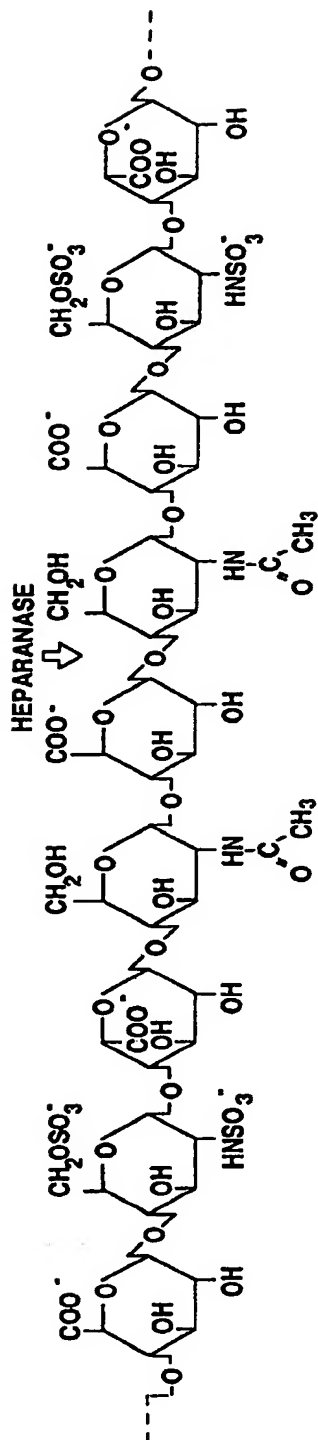
Fig. 8
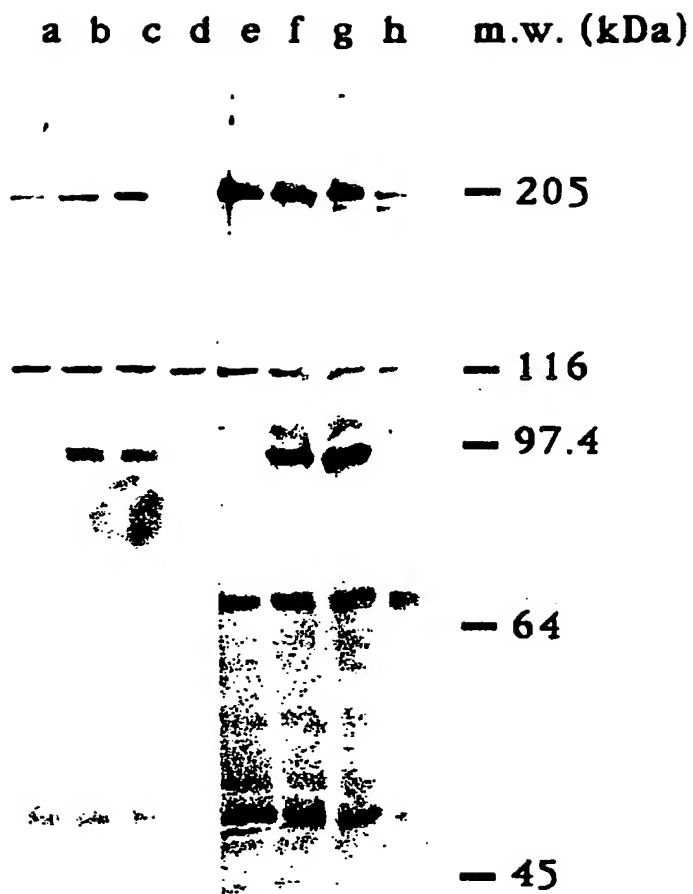
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a b c d e f g h   m.w. (kDa)

— 205

— 116

— 97.4

— 64

— 45

FIG. 9

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FIG. 10a

FIG. 10b

FIG. 10c

FIG. 10d

FIG. 10e

FIG. 10f
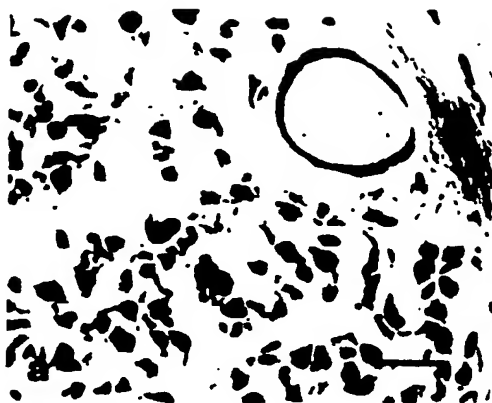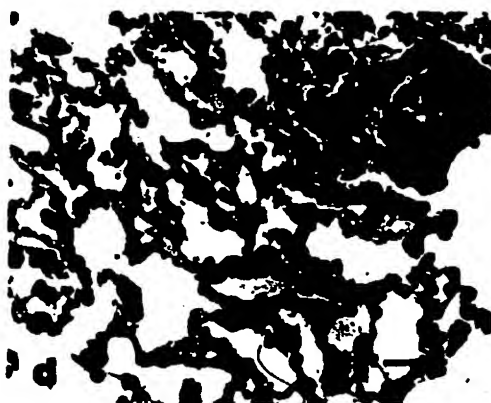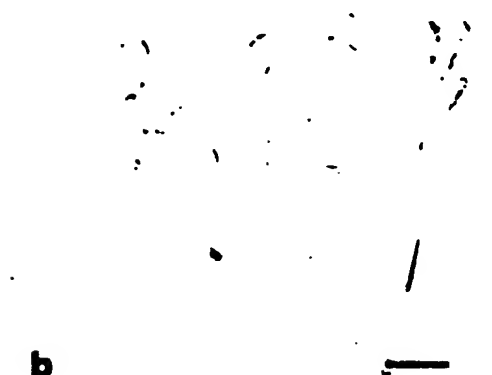
FIG. 11a

FIG. 11d

FIG. 11b
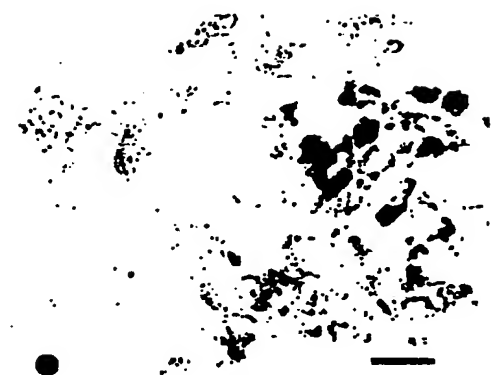
FIG. 11e
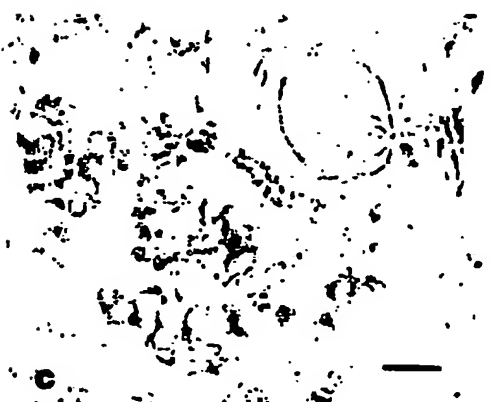
FIG. 11c

FIG. 11f

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INTERNATIONAL SEARCH REPORT

International Applic: No   PCT/US 91/03832

I. CLASSIFICATION OF SUBJECT MATTER   (If several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5   G 01 N 33/573   C 07 K 7/08 // G 01 N 33/574

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols		
Int.Cl.5	G 01 N	C 12 N	C 07 K

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[*]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	EP,A,0244932 (BOARD OF REGENTS THE UNIVERSITY OF TEXAS SYSTEM), 11 November 1987, see abstract, page 5, lines 6-12; page 8, line 24 - page 9, line 30; claims 37-39,53-59	1,2,4-21,23-26
X,P	International Journal of Cancer, vol. 45, 15 June 1990, Wiley-Liss, Inc. (US), Li Jin et al.: "Immunochemical localization of heparanase in mouse and human melanomas", pages 1088-1095, see the whole article	1-9,27-32
X,P	WO,A,9102977 (HADASSAH MEDICAL ORGANIZATION) 7 March 1991, see the abstract; claims 48-50 -/-	1,2,4-9

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
09-10-1991	2. OCT 1991
International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	MISS T. TAZELAAR

Form PCT/ISA/210 (second sheet) (January 1985)

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	III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)	
Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	Journal of Cellular Biochemistry, vol. 36, 1988, Tumor Progression and Metastasis, Alan R. Liss, Inc. M. Nakajima et al.: "Heparanases and tumor metastasis", pages 49-59, see the whole article (cited in the application)	1,5,6,8

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ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.

US 9103832
SA 49811

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 21/10/91
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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A- 0244932	11-11-87	US-A-	4859581	22-08-89
		AU-B-	609386	02-05-91
		AU-A-	6987087	17-09-87
		JP-A-	62265998	18-11-87
WO-A- 9102977	07-03-91	AU-A-	6336490	03-04-91

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